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HISTOLOGY—I MICROSCOPY

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A COURSE
IN
NORMAL HISTOLOGY

PUBLISHERS' ANNOUNCEMENT

RUDOLPH KRAUSE—HISTOLOGY

We offer this book to teachers and students alike as a proper guide in Histology.

Professor Krause strikes a keynote in his preface to the book. The study of Histology should go hand in hand with that of Anatomy. The one cannot be separated from the other nowadays without serious injury to the student.

The microscope is the hand-maid of anatomy. For this reason it has been deemed expedient to publish the book in two separate sections.

The first part is simply a guide to the technique of microscopy, and may be used by students of medicine as well as by those who pursue subjects of science foreign to medicine.

The second part deals exclusively with histology appertaining to medicine. Either part may be obtained independent of the other.

In bringing this work before the public it is the author's aim to give to the student a book of reference which is both practical and theoretical, as well as to furnish the teacher with a text-book which provides him with a detailed, yet clear, concise and methodical guide through the course of Microscopy and Histology.

So far as the student of medicine is concerned, he will find therein much valuable information appertaining to the microtechnique which must necessarily be taught to the exclusive student of Pathology. The subject of microtechnique is in most all English-written books on this topic treated but very briefly. This work not only teaches the student, in a most thorough fashion, the theory and manipulation of the microscope, but enables him to acquaint himself with *all* the methods employed in preparing a specimen for microscopical examination from start to finish.

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NEW YORK, 1123 Broadway.

A COURSE

IN

NORMAL HISTOLOGY

A GUIDE FOR

PRACTICAL INSTRUCTION IN HISTOLOGY AND MICROSCOPIC ANATOMY

BY

RUDOLF KRAUSE

A. O. PROFESSOR OF ANATOMY AT THE UNIVERSITY OF BERLIN

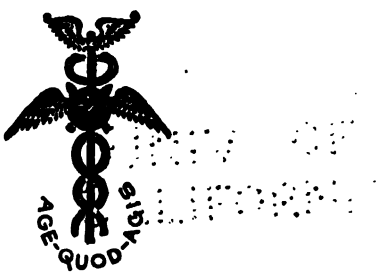
TRANSLATION FROM THE GERMAN BY

PHILIPP J. R. SCHMAHL, M.D.

NEW YORK

WITH 30 ILLUSTRATIONS IN TEXT AND 208 COLORED PICTURES, ARRANGED ON 98 PLATES AFTER THE ORIGINAL DRAWINGS BY THE AUTHOR

PART I.



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IN GRATEFUL ESTEEM

BY THE AUTHOR

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Preface

The brilliant progress made in microscopy during the last three to four decades has gradually brought with it a total change in the course of histologic instruction. In former days the student himself prepared his own microscopic specimens with razor and needle and with the aid of a few solutions for maceration, hardening and staining. To-day, after recognizing the inefficiency of such methods, this work has almost entirely been taken from the student's hands. Instead he receives from the assistant or preparator more or less immaculate specimens, preserved, cut and stained; some only for a single observation, others as his property. The practical course has been replaced by one of demonstration; at any rate in all its essential phases.

Special courses have been designed for instruction in the technique of microscopy, with the result that such courses have but a minimum percentage of attendance. By far the majority of our students enter the clinical semesters without being able to prepare a useful microscopic specimen. Not until later, during a course in pathologic histology or in the laboratories of clinics, will they learn those micro-technical manipulations so essential to them as practitioners.

This, in my estimation, is not a normal state of affairs. Every medical student, upon entering the clinical semesters, should be able to master micro-technical methods to such a degree that he is able to prepare a good specimen for microscopic examination from any organ given to him. This will be possible only when the course in histology occupies that rank of which it is deserving; that is to say, when the student ceases to be granted the meagre allowance of only four, at the utmost six hours per week; but, on the contrary, as has already been attempted in several places, when he is permitted to make use of one entire summer semester to such advantage as was proffered heretofore during two preparatory winter semesters. Then, such courses as "Technical Instruction," "Advanced Course," or whatever they may be called, will be dispensed with. The average medical man must receive an adequate technical education in histology. The scientist should seek the laboratory.

During the time that the student does practical histologic work, he should be bound to improve his theoretical knowledge also, which at the very best, those who know will admit, leaves much room for improvement under present conditions Without doubt the student takes a greater interest in a specimen

made by himself than in one already prepared, whether presented to him or only loaned.

The aim of this book is to direct the student to a practical education in histology and to microscopic anatomy, by the simplest methods. Most of the specimens described can be prepared from beginning to end by the student himself, if he employs the technique of freezing, which has long since acquired the title of "standard" in pathologic institutions, but was wofully neglected by anatomists in the past.

By dividing a larger class of students into separate divisions, each one of which being supplied with a freezing microtome, every participant will find ample opportunity during the summer period to attain experience in microtomy. Furthermore, it will be possible for every student of each single group to go through all the methods of embedding in paraffin and celloidin at least once, from beginning to end, during the semester. Moreover, it must be understood that unstained paraffin sections are given out on isinglass. The staining must be done in all cases by the student himself, whether frozen, paraffin or celloidin sections are used.

It goes without saying that staining of living tissues with methylene blue, injection of blood-vessels and lymph-ducts, and similar complicated technical manipulations cannot at once be performed by the student himself. He will learn by witnessing these procedures. And this, too, can be easily accomplished when the classes are grouped into subdivisions.

I assume, against custom, that the student is supplied with a modern microscope having a condenser and homogeneous immersion. The latter, of course, may be replaced by high power dry system to some extent. I hold that what has been an established factor in the bacteriological course hitherto, should also be made possible in histology. When we look at the time-honored *Schicks, Ploessls, Hartnacks*, etc., employed in some of the histologic courses, we cannot help but notice that we have not kept in step with other lines of our science. Granted that the experienced eye can see most things well enough even with the aid of such instruments, we should not add to the difficulties which confront the beginner, the additional tax of inadequate tools. On the contrary we should seek to assist him in his studies by placing at his disposal the most perfect instruments. It is of the greatest importance to use the best drawings that can be made of the specimens, and every student should be obliged to make faithful sketches of his slides. Microscopic drawing is not an art in the strict sense of the word. In order to stimulate drawing by students, I have undertaken the task of sketching every preparation myself. These drawings, no more than those of students, are not intended nor expected to be works of art, but solely true representations of the microscopic findings. Each sketch has been prepared with the aid of *Abbé's* drawing apparatus, and the enlargement is marked

on every one. The fraction, which is often annexed, indicates how much the original has been reduced in the reproduction.

To obtain the truest image possible of the original drawing, I have employed the autotype, which has attained such a standard of perfection to-day as to bring out the finest details of a microscopic image. The cuts have been made in a most excellent manner by the *Angerer and Goeschl Art Printing Co.*, of Vienna. The printing was done by *Christoph Reisser's Sons*, also of Vienna. To both firms I am indebted for their readiness in giving my wishes the closest attention, and also for the superior execution of the plates.

A text is furnished with every drawing. It precedes each plate, explains the technique, and gives a brief description of the specimen. If the latter is not confined entirely in all the details to the drawing, but deals also with other matters, not depicted, it does not follow that this guide-book pretends to represent a text-book of histology, which it cannot be any more than the histologic course can be an adequate substitute for a course of lectures in histology and microscopic anatomy; they supplement one another, but the one cannot replace the other. For that reason it is desirable that text-books on histology should cast off that technical ballast which they carry in contradistinction to other departments.

In fine I deem it a pleasurable duty to heartily thank my publishers. They have met my wishes in every detail and have spared neither pains nor money in order to equip this volume in the most lavish manner.

BERLIN. RUDOLF KRAUSE.

Translator's Preface

At the request of Mr. F. J. Rebman, I have undertaken the translation of Rudolf Krause's Histology into the English language. I have adhered to the German text as closely as possible, without, however, making sacrifices to idiomatic expressions. My aim has been to make the book as useful to the student of medicine in English-speaking countries, as is compatible with the spirit in which the author has written it.

Professor Krause seeks to raise the level of instruction in Histology to that obtained during recent years in other departments of medical education. He deserves encouragement in this praiseworthy endeavor, and the authorities entrusted with the education of aspirants in medicine at the various colleges are supporting him in his claim.

It affords me pleasure, therefore, to be enabled to place at the disposal of teachers and students of Histology an English version of Professor Krause's teachings, and I sincerely trust that the book will find a proper place in the curriculum of the medical schools of all countries in which the English language is spoken.

I take pride in expressing my obligation to Mr. Rebman for the aid he has given me in preparing my manuscript, especially in the translation of the preface by the author. I likewise cheerfully acknowledge the assistance derived from the perusal of Edward Bausch's "Manipulation of the Microscope."

P. J. R. Schmahl, M.D.

New York City.

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UNIV. OF CALIFORNIA

Introduction

THE AIM OF A COURSE IN HISTOLOGY

The object of the histologic course is a dual one: on one hand it should serve as a completion of the theoretical lectures on general and systematized anatomy, i.e., it should demonstrate *ad oculos* what the student has been told of the microscopic structure of the human body and enable him to understand, recognize and differentiate for himself the organs and the tissues composing them. The daily practical work with the microscopic preparation should give him a fixed and definite idea of the normal structures of the various organs, in order to enable him in later semesters to recognize and diagnose the pathologic changes found therein.

If this were the only object, it would suffice to assign a number of good specimens to the student, to discuss them thoroughly with him, and to let him reproduce them with the pencil on paper. But such is not the case. Our course of instruction has a secondary aim, equally important and a good deal more practical, viz., to initiate the student in micro-technique, which latter is of the greatest importance for advanced study as well as general practical education. At the present day the microscope is probably the most important and necessary requisite of a medical instrumentarium. A thorough training is needed for handling it in the proper manner, and the basis of this training rests with the histologic course, at the end of which the student should be thoroughly familiar with the microscope and its use.

But this is by no means sufficient. The use of the microscope presupposes the knowledge how to prepare a specimen for examination, and for this reason it becomes necessary for the student to acquire skill in what is commonly termed ***microscopic technique*** or ***micro-technique.*** This, however, is an extremely large field, which requires years of study to master, and no sane person will ask the student to familiarize himself with all the innumerable methods and details of procedure. Nevertheless he should attain that amount of technique to enable him to prepare, from any given organ, a specimen which clearly shows its structural peculiarities.

To be a guide in accomplishing this end is the object of this volume. Primarily it will familiarize the reader with the microscope and its use. Furthermore, it will bring to his attention the most important reagents, stains, and microtechnical methods; and lastly, in the Special Part it will give detailed instructions for the production of good specimens, suitable to the study of each tissue and organ, and to elucidate in words and pictures what may be learned from these specimens.

Untiring pains have been taken to reproduce the most characteristic parts of a preparation. The cuts should stimulate the reader to draw specimens for himself. If the first endeavors should prove unsatisfactory, there is hardly anything else that will be so easily and quickly learned with a little perseverance and a bit of adroitness, than is microscopic drawing, which does not, as the beginner generally fears, necessarily require any special talent. Not until drawing it, do we fully appreciate a specimen and learn all about it, and our drawing, when completed, will forever remain vivid in our memory, an indelible picture.

GENERAL PART

The Microscope

The elements constituting the human and animal organism are of such minute size that in most instances they cannot be recognized by the unaided eye; the necessity arises therefore, to employ certain instruments which will present to our eye a magnified image. The knowledge of such agents dates far back in history. The fact that lenses, i.e., transparent spherical bodies, will under certain conditions magnify the articles viewed through them, has been known for ages.

Lenses as a Means of Magnifying.

In ancient times **lenses** were made from more or less valuable natural crystals. This is on rare occasions done even to this day; but as a rule lenses are now made of artificial glass of a fixed chemical combination, for it has been found that the composition has a great influence on the optic properties, i.e., on refraction and dispersion power. Lenses with moderate dispersion and greater refraction power are designated as ***crown glasses,*** those with high dispersion as ***flint glasses.*** In the preparation of the latter a large percentage of lead oxide is added to the liquid glass. They also contain arsenic, manganese, and, of course, silicon dioxide in small amounts. Crown glasses, on the other hand, contain also a good percentage of silicon dioxide, the alkali metals, alkaline earth metals and boric acid anhydride. It follows that flint glasses are, as a rule, heavier than crown glasses. The dispersion power of the flint is in direct proportion to its weight, i.e., amount of lead contained in it.

Classification of Lenses.

According to their form lenses may be divided into two main divisions: those which are thicker in their centre, the optic axis, than on the periphery, and those in which the thickness decreases from periphery to optic axis. The

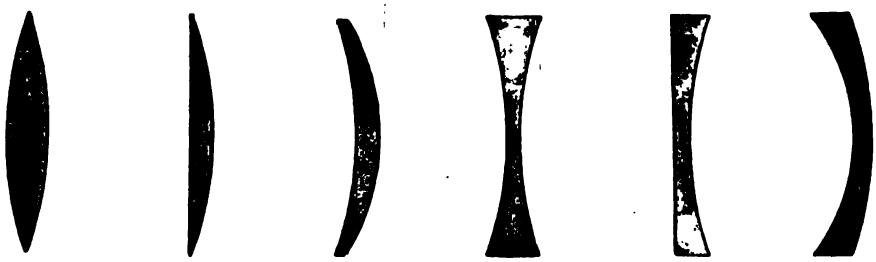


FIG. 1.
Positive Lenses.

FIG. 2.
Negative Lenses.

former are called ***positive*** or ***convex lenses,*** while the latter are known as ***negative*** or ***concave lenses.*** Under the positive lenses we again distinguish between biconvex, plano-convex and positive meniscus (Fig. 1). All parallel

rays falling on a positive lens are known to unite in a point on the other side of the lens, situated on the optic axis and called the ***focus.*** The distance of the focus from the lens is termed ***focal distance.*** Parallel rays falling on a negative lens are dispersed in such a fashion as if emanating from a point this side of the glass. Here also we speak of focus and focal distance, but prefix them with negative or virtual. Among the negative lenses we also speak of bi-concave, plano-concave and negative meniscus (Fig. 2).

Image-Formation Through a Biconvex Lens.

According to known optic laws a biconvex lens furnishes an inverted real picture of an object, situated beyond its focal distance, i.e., in our case (Fig. 3)

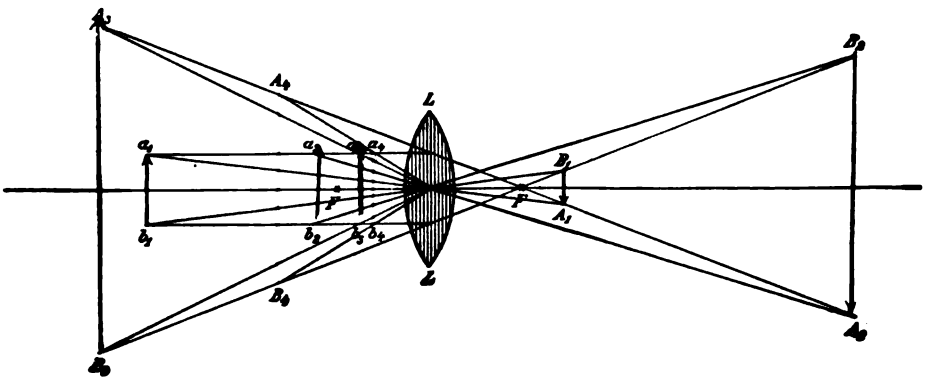


FIG. 3.

Reproduction through a Biconvex Lens.

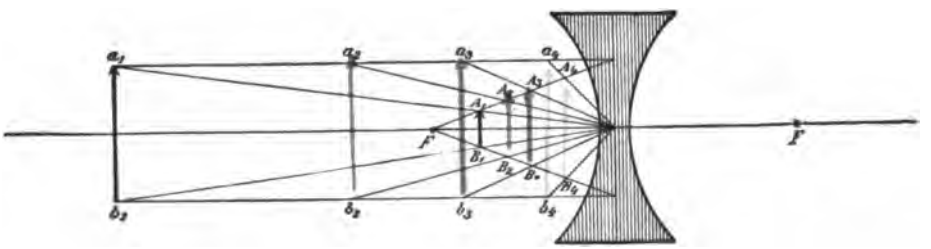


FIG. 4.

Reproduction through a Biconcave Lens.

an image situated to the right of the lens ($a_1\ b_1$—$A_1\ B_1$). If the object approaches the focus, the picture recedes from the lens, becoming larger ($a_2\ b_2$—$A_2\ B_2$), and finally, when the object occupies the focus, is lost ad infinitum. If the object encroaches on the focal distance itself, we get an erect, enlarged, virtual image, situated to the left of the lens ($a_3\ b_3$—$A_3\ B_3$). The closer the object approaches the lens the smaller the picture will be, though always remaining larger than the object itself ($a_4\ b_4$—$A_4\ B_4$).

The Biconcave Lens.

Much simpler is the action of a biconcave lens. It gives us under all conditions a reduced erect image, which becomes larger as the object is approximated to the lens (Fig. 4).

Magnifying.

If we want to distinctly perceive with our eyes a picture, reflected by a lens, it becomes necessary to establish a distance between image and eye of approximately 250 mm, i.e., to place it in distinct visual distance. The ***magnifying power*** furnished by a certain lens is dependent upon its focal distance and is found by dividing the distinct visual distance of the particular observer by the focal distance, both expressed in millimetres. The focal distance in turn is dependent upon the curvature of the planes of the lens and the refraction index of the particular glass. For example, we find a focal distance of the lenses, represented in Fig. 1, of 50 mm for the biconvex, 100 mm for the plano-convex and 150 mm for the meniscus, presuming the refraction index to be 1.5. These lenses, therefore, would possess a magnifying power of 5, 2.5 and 1.6 respectively. With the negative lenses of Fig. 2, on the other hand, we get a focal distance of —50 mm for the biconcave, —100 mm for the plano-concave and —150 mm for the meniscus. These lenses, therefore, will reduce correspondingly.

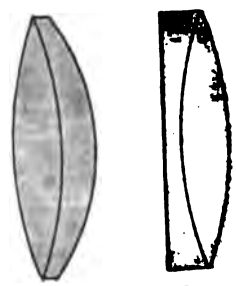


FIG. 5.
Compound Lenses.

Very often two or more lenses are combined and cemented together by a transparent resin in a fashion that they are intimately approximated. They may be considered as one lens with a reciprocal focal distance equal to the sum of the reciprocal focal distances of the component lenses; e.g., the biconvex lens will gain in focal distance by adding a positive meniscus (Fig. 5, *a*), while it will lose when combined with a plano-concave lens (Fig. 5, *b*). Again, the components may be made of the same sort of glass or, what is done much more frequently, two different kinds of glass with different refraction and dispersion power are selected; e.g., a combination is made of a plano-concave lens of flint glass and a biconvex crown glass.

After what has been said it would seem that the strongest enlargement could be obtained by the use of a simple or a combined positive lens, since it would be only necessary to use lenses with a corresponding short focal distance. A biconcave lens of 1 mm focal distance, i.e., 1 mm curvature radius, should give us an enlargement of 250 magnifying power. Practically this cannot be accomplished, since such small lenses, aside from the difficulty of their manufacture, are too weak, the distance between object and eye would become too small, and finally such lenses would possess the two great shortcomings of every spherical, refractive plane, namely, spherical aberration and chromatic aberration, to such a degree as to make them useless in practice.

Spherical Aberration.

By ***spherical aberration*** we understand that peculiarity of every spherical, refractive plane by which only those of the parallel rays of light that

are nearest the optic axis meet in the focus, while those nearer the periphery are united nearer the lens, approximating the latter more and more the nearer they are to the periphery.

Chromatic Aberration.

Since red rays are refracted less than blue, the former will have a greater focal distance than the latter. This phenomenon has been called ***chromatic aberration.***

To avoid spherical aberration the peripheral rays are deflected, but also can be obliterated by correct construction and position of the lenses. For instance, a biconvex lens with equally curved planes will show a greater aberration than one in which the curvature radii varies, bearing a fixed relation to one another. A plano-convex lens will give less aberration than a biconvex, if the plano-surface of the former is directed toward the incoming rays. To avoid chromatic aberration the optician combines lenses of different dispersion power. He will, for instance, correct the dispersion power of the biconvex

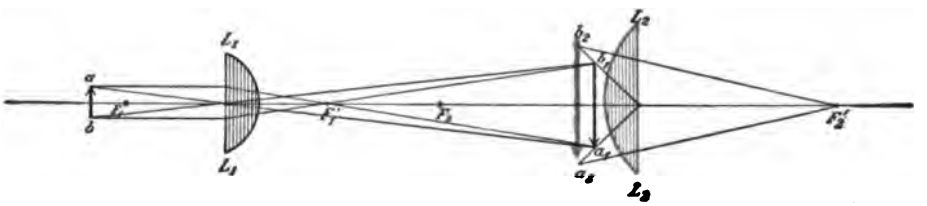


FIG. 6.
Reproduction by Two Plano-Convex Lenses.

crown-glass lens (Fig. 5 b_1) by a plano-concave lens of flint glass, thereby sacrificing, however, the refractive power.

The Combined System.

In practical work one never goes below a focal distance of 10 mm in a single lens, hence, with such a lens, no enlargement greater than 25× is ever obtained. If greater power is desired, two or more lenses, separated a certain distance from one another, are used. Such a combination we find illustrated in Fig. 6. Here we have a plano-convex lens (L_1) of 20 mm focal distance. The object $a\ b$ being situated right in front of the anterior focus, the lens projects a reversed enlarged real image $a_1\ b_1$. This image lying again within the focal distance of a second plano-convex lens (L_2) of 40 mm focal distance, a second, still more enlarged, virtual and also reversed picture (referring to the object) $a_2\ b_2$ is produced. Hence, by projecting the image of a primary positive lens within the focal distance of a second positive lens, we are enabled to produce a second enlargement of the image, and from what has been said it is evident that this enlargement will become greater as the distance between the two lenses is increased, i.e., dependent on how near we bring the first image to the anterior focus of the second lens.

Course of Rays in the Compound Microscope.

In Fig. 7 things are still more complicated. Here we have taken three lenses into our optic system: the plano-convex L_1 (20 mm), the convex-plano

L_2 (40 mm) and the convex-plano L_3 (30 mm). The distance between the second and third lenses has been so chosen as to amount to half the sum of their focal distances, viz., 35 mm. The size of the object and its distance from the anterior focus of the first lens being the same as in the previously mentioned case, the image obtained would also be the same, if lens L_2 had not been interposed in the course of rays, converging the rays and producing the much smaller image a_2 b_2, instead of a_1 b_1. This second picture is projected within the focal distance of the much more curved lens L_3, which finally furnishes the greatly enlarged image a_3 b_3.

A similar arrangement we find in our ***compound microscope.*** Here, too, an enlarged reversed real image of the object is projected through a lens or rather a system of lenses, the focal distance of which ranges between 1.5 and 40 mm, since the object is situated closely anterior to the focus of this system. The latter, being so proximal to the object, is termed the ***objective*** or ***object-glass.*** The lenses L_2 and L_3 are united into a system, which, being

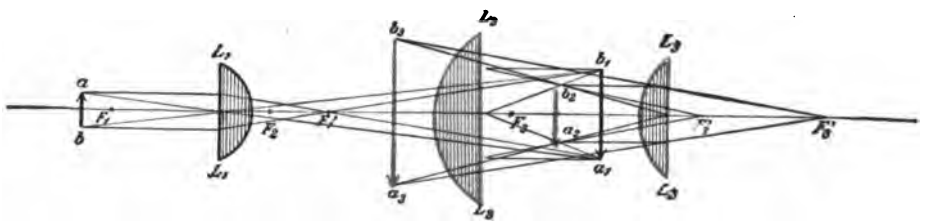


FIG. 7.

Course of Rays in the Compound Microscope.

proximal to the eye of the observer, has been termed the ***ocular*** or ***eye-piece.*** The lens L_2 again is called ***collective lens,*** while L_3 receives the name ***eye-lens.*** Here, too, as in the previous case, the distance between the eye-lens and the objective lens equals about half of the sum of their focal distances. On the other hand, in the microscope the eyepiece is much more distant from the object-glass, as in our given example, the distance of the upper focus of the object-glass from the lower one of the eyepiece being 160 mm. This distance has been designated as the ***optic*** or ***reduced tube-length*** ($\triangle$), and is chosen so as to project the image produced by the eye-lens of the eyepiece at a distance of 250 mm, i.e., in the distinct visual distance of the observer.

Magnifying Power of the Compound Microscope.

The enlargement produced by such a combined microscope is always easily figured out if the focal distances of the object-glass (F_1) and the eyepiece (F_2) are known, viz.:

$$N = \frac{250}{F_1} \cdot \frac{\triangle}{F_2}$$

After these initiating remarks let us proceed to the description of a microscope, which seems especially adapted for our purpose.

Description of the Microscope.

Our instrument (Fig. 8) rests on a horseshoe-shaped ***foot*** or ***base,*** surmounted by the ***barrel*** or ***body.*** Generally we find in the latter, right over

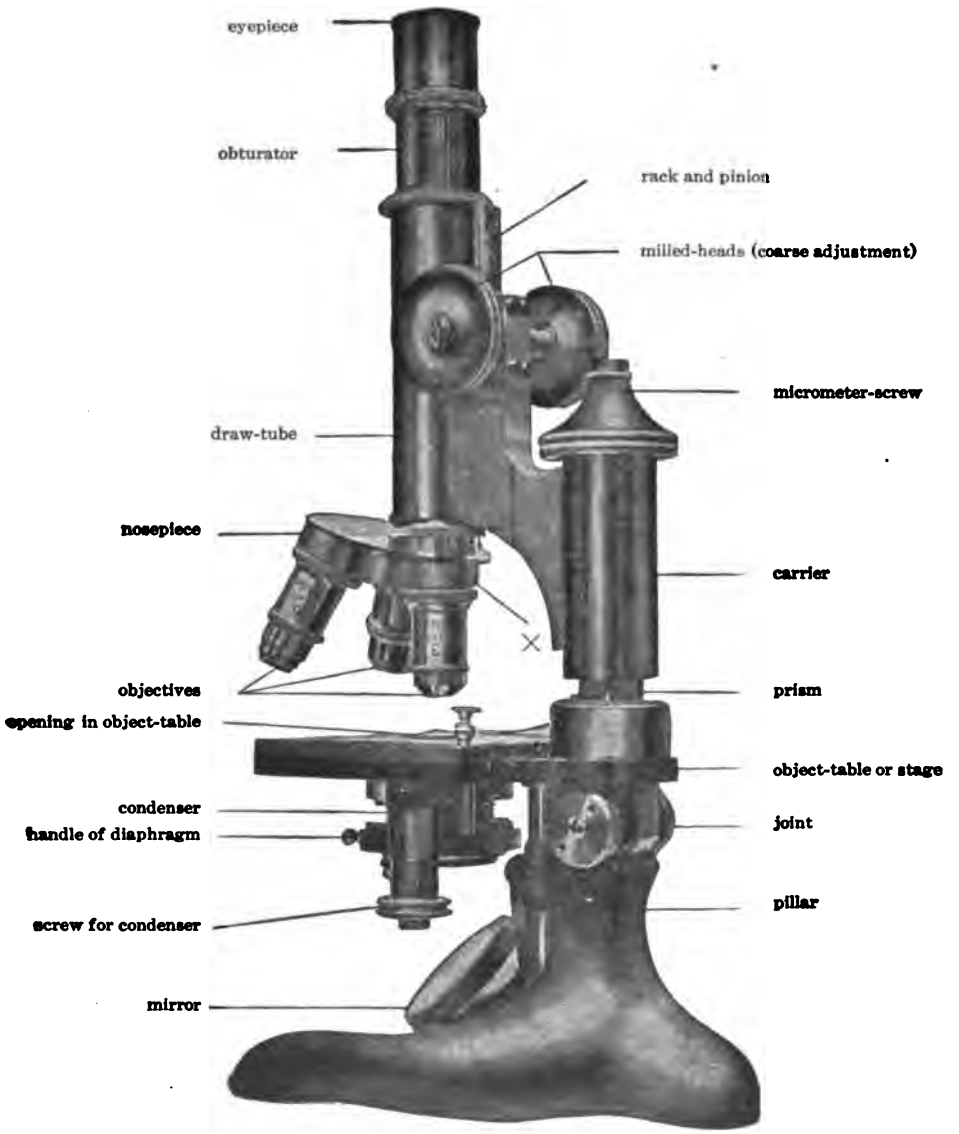


FIG. 8.
The Microscope.

the foot, ***a joint,*** which allows an oblique position of the instrument, thus permitting of a more convenient examination in the sitting posture. Closely above this joint is the square ***stage,*** intimately connected with the barrel and having a round opening in its middle. Here the specimen, fixed by ***clips,*** is deposited

for examination. The table divides the microscope into a lower and upper part.

On the inferior surface of the stage a projection is found, the ***condenser,*** a system of lenses set in metal, for illuminating purposes. By means of a screw the condenser can be adjusted. By turning it to the right, you approximate the apparatus to the stage, gradually introducing it through the opening in the stage, until the upper surface of its superior lens is on a level with the upper surface of the table. Turning to the left, the condenser will recede from the table, and finally swings out to the left.

On the lower portion of the condenser two knobs are seen, one serving to open and close the ***diaphragm,*** the second being attached to a swinging ring, into which colored (e.g., blue) glass plates may be inserted, such as are necessary, when working in artificial light.

Below the condenser the ***mirror*** will be found, a silvered double mirror, having one plane surface on one side and a concave on the other. It is connected with the pillar by a movable lever and suspended in a metal crescent so as to allow of any position.

Above the object-table the pillar continues as a trigonal ***metal prism,*** visible only for a few millimetres, as it is enveloped by a hollow metal cylinder, the barrel ***carrier.*** The hollow of the carrier corresponds exactly to the size of the prism, so that the two are in intimate relation. On top the carrier is surmounted by a hoodlike screw head, the head of the ***micrometer-screw,*** in front it is elongated into an armlike projection, which supports the barrel.

The ***barrel*** or ***tube*** is a hollow brass cylinder. Above it is mounted by a ring, through which glides a second graduated tube, the ***draw-tube.*** The scale is graduated in millimetres, ranging from 140 to 200 mm or 14 to 20 cm, starting on top with the smallest figure and ending below with the largest. These figures represent the tube length, figured from the inferior outlet of the barrel, to which the objective is attached, up to the superior border of the draw-tube, on which the eyepiece rests. If we draw out the draw-tube we increase the distance between objective and eyepiece, and hence between their respective foci. Thereby the magnifying power of the microscope is increased.

This fact may be made use of when employing a weak objective, but is objectionable with the stronger object-glasses, as the latter have been construed for a certain tube length, viz., 160 mm. We must also take into consideration that in our instrument a nosepiece, 8 mm thick, is inserted between barrel and objective, which must be allowed for, so that for all the stronger enlargements the barrel must be drawn to 152 mm, i.e., the draw-tube must register 15.2.

Body and arm are connected by rack and pinion, the rack forming part of the body posteriorly, the pinion part of the arm, the teeth engaging in recesses on a transverse rod, which has screw-heads on either end, the ***coarse adjustment.*** By turning these knobs the barrel is lowered or raised, thus approximating or receding from the stage.

On the lower end of the barrel the so-called ***nosepiece*** is found, for the purpose of quickly changing objectives. It consists of a metal plate having three arms, each arm being provided with a threaded ring, the whole revolving

on a common axis. The plate itself has a threaded hole, by virtue of which it is attached to the barrel. When screwed in place the three-armed piece will revolve around the plate in such fashion as to fit the rings exactly to the lower barrel opening. A spring (×) in the back of the nosepiece indicates whether the position is accurate, so that one may look right through the barrel and one ring, the other two being covered by the plate.

The ***eyepiece*** is attached to the upper aperture of the draw-tube, while below, the ***objectives*** are screwed to the rings of the nosepiece. The latter should be placed in consecutive order, viz., assuming objective *3* to be in place, first objective *6* and then objective $^1/_{12}$ should follow, when the nosepiece is turned to the right.

Several of the parts of the microscope spoken of require a more thorough discussion, principally the ***objectives,*** the ***eyepieces,*** the ***condenser*** and finally the ***micrometer-screw.***

THE OBJECTIVE

The Significance of the Objective.

As already explained in our general discussion of refraction of rays in the compound microscope, the purpose of the objective is to furnish a real, reversed, enlarged image of the object to be examined. The latter simply being enlarged again by the eyepiece, it must needs be free of all refractive errors, free of all spherical as well as chromatic aberration. This leaves no question that the objective is the most important part of the entire microscope, being of much greater consequence than the eyepiece, since we can merely enlarge the objective image considerably by a strong eyepiece, without bringing any new elements into the microscopic picture. A structure not previously revealed by the objective will never be disclosed by an eyepiece, no matter how strong the latter may be.

Structure of the Objective.

In order to comply with all the requirements, the objective in the compound microscope cannot, as we have supposed in our earlier discussion, be represented by a single lens, but is composed of a ***series of parts,*** arranged in a common metal setting in such a fashion that all are exactly concentric, i.e., all lie in the same optic axis.

As a rule, the different members are connected to one another at a fixed distance, although in rare instances the distance may be altered within certain limits.

Each member is attached to the lower end of a brass tubule, and these tubules, sometimes separated by an interposed metal tube, are screwed one into the other. According to the number of lenses we have double, triple and quadruple systems. Commonly each successive member is larger than the preceding, making the first member, the so-called ***anterior*** or ***front lens,*** the smallest of all.

This front lens is the principal or sole agent in ***magnifying,*** while the remaining lenses or ***correction lenses*** have as their object the clearing out of errors from the image furnished by the front lens. Hence the size of the front

lens will give us an estimate of the power of the entire objective, so that we are able to say that, the larger the front lens is, the lower will be the power of the objective. Since low-power objectives have a greater focal distance than high power, the object, however, necessarily being without the focal distance of the objective, it will be seen that during a sharp focus the distance between object and front lens of objective, the so-called ***free object distance,*** is greater in low-power objectives than in high. For example, with an objective of 42 mm focal distance we will get an object distance of 40 mm, while one of 1.8 mm will have only 0.17. In the latter, therefore, the front lens must be brought very near to the object.

In low-power objectives this front lens is mostly composed of several single lenses; in high-power object-glasses, however, there is generally one single plano-convex (hemispherical) lens, the plane surface being turned toward the object. Most of the weaker objectives are doublets, one combination lens following the combination front lens. Here both members take part in the magnifying as well as correcting process. Medium objectives have two combined correction lenses above the front lens, the latter furnishing the power, while in the most powerful object-glasses we find between front lens and the correction systems a fourth simple lens, either plano-convex or a positive meniscus.

Achromatic and Apochromatic Objectives.

The individual systems, and the lenses composing them, are made of different artificial glass compounds, each possessing its own refractive and dispersive qualities. Recently objectives have been constructed with lenses made partly of a single mineral, namely ***fluor spar.*** As regards refractive index, this mineral only equals crown glass, but it excels all artificial glasses in its dispersive power. By the use of fluor spar the greatest color purity of the microscopic image has been obtained. Objectives made of glass solely are known as ***achromatic,*** those in which fluor spar is used as ***apochromatic.*** For our purpose the latter may be disregarded, being much more expensive than the former on account of the high-priced raw material, without yielding much better results for our work.

Oil Immersion Objectives.

Finally, there is one more very important point to deal with. Heretofore we have always assumed that the rays emanating from the object enter the front lens directly through the air. In reality this is not so, as in most cases our object is covered with a thin plate of glass, the cover-glass. The rays of light therefore are first refracted by this cover-glass before reaching the front lens or, to be more exact, they emanate from the object, pass through the cover-glass, thence into the air and finally enter the front lens. The sealing medium possessing about the same refractive index as the cover-glass, the rays of light emerging from the object will pass the cover-glass unrefracted, but become refracted at the surface of the cover-glass, when the following takes place: since they pass from glass to air, they are everted from the optic axis, so that the angle of exit is greater than the entrance angle, hence a much smaller part of the light-rays enters the objective than we have heretofore supposed. This fact is not of great importance when dealing with objectives

having a large front lens, i.e., low-power objective, but is very noticeable when a small front lens is used. This condition is illustrated in the right half of Fig. 9, while the left half shows the remedy for it. All we have to do is to interpose between front lens and cover-glass a medium of the same refractive index as the latter, and the ray *a b*, otherwise refracted, will now reach the objective unrefracted.

The interposing of such a medium, which necessarily must be liquid, is done without difficulty, since the high-power objectives have but a small object distance. A drop of condensed *cedarwood oil* is placed on the cover-glass, the barrel is lowered so far as to allow the front lens to dip into the drop and thus a homogeneous connection between object and objective is effected. Such objectives are called ***homogeneous immersion objectives*** or ***oil-immersion lenses,*** and are to be differentiated from immersion objectives, where

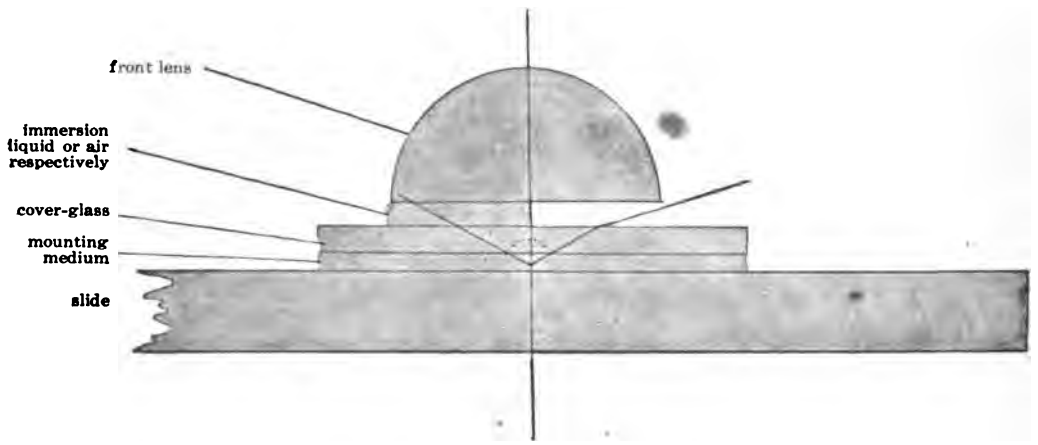


FIG. 9.
The Effect of Immersion.

no oil but some other liquid is used as a medium. We thus hear of immersion systems, where ***water*** is used, viz., a liquid of lower refractive index than oil, or those in which ***monobrome naphthalin*** is the medium, the latter of a higher refractive power, which is used when a front lens of strongly refractive flint glass is engaged.

Angular Aperture

From previous remarks it will be seen that an immersion lens is superior to a dry lens of the same focal distance and aperture, since in the latter the available aperture of the objective is not fully utilized. The ***angular aperture,*** or, in short, the ***angle*** of an objective, are terms used to designate the angle, having its apex at the central point of the object, its arms extending to the periphery of the front lens of the objective, when the object is in sharp focus. Its size is dependent upon the free object distance of the objective and the diameter of the front lens. The medium separating objective and cover-glass must also be taken into consideration when calculating the angle. The figure obtained when considering both, is the ***numerical aperture*** found by

multiplying the refractive index of the interposed medium with the sinus of half of the angular aperture:

$$a = n \cdot \sin u.$$

Since in the dry system n equals *1*, the aperture in such is equal to half the opening angle. With water immersion this value must be multiplied with *1.33*, with oil immersion by *1.51*, and with monobrome naphthalin by *1.62*. As the angle u can never attain 90°, its sinus, and hence the aperture of every dry system, is always less than *1*. On the other hand a may be larger than *1* when immersion systems are used.

The Requisites of an Objective.

A good objective should possess the following qualities: 1. It must be ***achromatic,*** i.e., red and blue rays should unite absolutely. 2. A ***planatic image*** should be obtained. 3. All rays emanating from one point of the object should, as far as possible, be united in one point of the image, i.e., ***sharp pictures*** should be furnished; no blurring should take place. 4. It should furnish ***detail,*** i.e., the finest structures of our specimen must be reproduced.

The achromatic correction of the objective, as we have seen, is brought about by employing lenses of varying dispersion properties. To procure aplanasia and the greatest sharpness, spherical aberration must be, as far as possible, obliterated. As has been previously mentioned, the different members of the objective are made up of lenses of various refractive powers and thus the preceding member is corrected by the succeeding one. The optician at times even goes as far as to overcorrect such a member, subsequently balancing this overcorrection by the following lens. The detailing power finally is wholly dependent upon the aperture and is in direct proportion with the increase of the angle.

Objectives are marked by their manufacturers either with figures (Arabic or Roman) or with letters, giving a higher number or a later letter of the alphabet to the stronger objective, and vice versa. Immersion objectives, on the other hand, and also the briefly mentioned apochromatics, are known by their focal distance, the former expressed in inches, the latter in millimetres. Objective 1/12 therefore will possess a focal distance of 1/12 inch, i.e., 2.1 mm.

Objective 3.

After these general preliminaries let us consider more closely those objectives which are of special interest to us (Figs. 10-12). Turning to figure 10 we find a section through objective 3. Here we have the type of a weak objective, consisting of only two lenses, these two members, however, being double lenses. The first member has a diameter of 6 mm and consists of a plano-concave flint-glass lens and a biconvex crown-glass lens, the focal distance being 15 mm. The second member, which is of the same combination, has a diameter and curvature radius 3 mm greater than the former, the focal distance being 29 mm with a distance from the first member of 14 mm. Thus the entire objective has a focal distance of 16.2 mm, a free object distance of

5.5 mm and a numerical aperture of 0.3. The objective alone furnishes an enlargement of 10.3, with the weakest eyepiece, however, one of 41; in the latter case it still covers a field of 2.1 mm diameter.

Objective 6.

Fig. 11 (objective 6) represents a system of medium strength. The three members composing it are placed in separate metal shells at distances 0.15, respectively 0.65 mm. The second shell is screwed over the third, while the first envelops both the former, being screwed to the base of the third, the upper extremity of which is in direct contact with the body proper of the objective. The first division consists of a hemispherical crown-glass lens of 3.5 mm focal distance and about the same diameter; the two succeeding members are made up each of a plano-concave flint-glass and a biconvex crown-glass lens. The focal distance of 12.5 mm in the second is raised to 17.6 mm in the third member, while the diameters are 6 and 7 mm, respectively, bringing the total

FIG. 10.
Objective 3 (Leitz).

FIG. 11.
Objective 6 (Leitz).

FIG. 12.
Objective 1/12, Homogeneous Immersion (Leitz).

focal distance down to 4.0 mm. The magnifying power of the objective itself is 48, with a free object distance of only 0.42 mm, while its angular aperture has increased to 0.82. With the weakest eyepiece one may obtain an enlargement of 192 with this object-glass, i.e., it is five times more powerful than objective 3, but the diameter of the visual field here only amounts to 0.48 mm, i.e., one-fourth of the objective 3.

Objective 1/12.

Fig. 12 acquaints us with a powerful objective, an immersion system. It is composed of four members, the first two being single, the last two double lenses. The distances are very minute: 0.02, 0.1, 0.15 mm respectively. Each division has its own shell; the setting of the first is made of aluminum. The various shells are attached to one another in such fashion that 1 is screwed to 2, 2 to 4, 3 to 4, and 4 to the body proper of the objective. The first member consists of a single hemisphere, made of crown glass with a diameter of 1.96 mm and a focal distance of 1.55 mm. The second is a positive meniscus with a focal distance of 4.92 mm, while the third and fourth are again plano-

convex flint-crown-glass double lenses of 11.14 and 29.28 mm focal distance respectively. The entire object-glass possesses a focal distance of 1.85 mm, a free object distance of only 0.17 mm and an aperture of 1.30. It has *per se* a magnifying power of 105, which increases to 420 with the lowest eyepiece, and a visual field of 0.24 mm is covered with this objective.

Objectives for Very Low Variable Enlargement.

Often a want is felt for a lower system with a correspondingly larger visual field. Objective *1a* is most adapted for that purpose. Since this type is new to us, we will give it brief mention here. It consists of two achromatic double lenses in a movable setting, so that they may be approximated or withdrawn from one another by means of a screw to the extent of about 5 mm. The front member consists of a biconcave flint-glass lens in back of a plano-convex crown-glass lens, and has a negative common focal distance of 14.8 mm. The upper member is made up of a plano-convex flint-crown-glass double lens of 24.0 mm focal distance. When both systems are at a maximum distance (30 mm), the common focal distance is 24 mm. When approximated as far as possible, this increases to 33 mm. Here the magnifying power proper falls from 3.1 to 2.0 and the object distance is thus increased from 2.0 to 14.0 mm, while the numerical aperture decreases from 0.07 to 0.05. With the weakest eyepiece a power of 8—12 is obtained with a visual field of about 10 mm diameter. The distance of the two lenses from each other can be read on a scale marked externally.

THE EYEPIECE—(Ocular)

The eyepiece consists of a metal cylinder fitting snugly into the draw-tube, the component lenses being mounted on either side. On its upper extremity we find a metal ring protruding, preventing the ocular from sliding through the tube.

The Huyghen Eyepiece.

There are various sorts of oculars, principally the ***Huyghen,*** the ***Ramsden,*** and the so-called ***compensation eyepiece.*** We will only consider the first. The *Huyghen* ocular (Fig. 13) is composed of two plano-convex lenses, one of which, the ***eye-lens,*** is placed at the upper, the other the ***collective lens,*** on the lower end, the convexity of both being toward the objective. Between the two lenses we find a ***diaphragm*** on the interior of the ocular.

FIG. 13.
Huyghen's Eyepiece (Leitz).

The eye-lens is always the stronger, having only half the focal distance of the collective lens. As previously mentioned, the distance between the two lenses is half of the sum of their focal distances. Hence these focal distances, and thus the total focal distance of the ocular, may easily be determined by measuring the distance, i.e., the length of the eyepiece. For example, the dis-

tance (e) of the two lenses in our eyepiece *III* measures 36 mm in round figures; using the equation: $e=\frac{f_1+f_2}{2}$ and granting that $f_2=2f_1$, f_1, *i.e.*, the focal distance of the eye-lens, must be 24 mm; f_2, i.e., that of the collective lens, will be 48 mm, and the total focal distance of the ocular, f, according to the formula $f=\frac{f_1 \cdot f_2}{e}$ will be found to be 32 mm. The lower focus of the ocular lies between its lenses, the upper externally above the eye-lens. It is of great importance, as far as the *Huyghen* ocular is concerned, that it is constructed in accordance with formula $e=\frac{f_1+f_2}{2}$ as nearly as possible, for it is due to that fact only, that, in spite of the eye-lens as well as the collective lens being simple non-achromatic, the image furnished by the objective is enlarged without aberration.

Significance of Eyepiece.

As we have previously observed, the image produced by the objective is first reduced by the collective lens, the latter collecting the rays coming from the former. The diaphragm is found in the plane, where this reduced real image is produced, which latter may be demonstrated as follows: Focus some object, and after removing the eye-lens place a properly cut piece of thin tissue-paper on the diaphragmatic opening; if the collective lens is now also removed, a much enlarged image will be projected on the diaphragm, which can be focused sharply by adjusting the draw-tube.

Thus our real image is simply enlarged by the eye-lens and brought into exact visual distance of the observer. At the same time, of course, the rim of the diaphragm is projected, so that our field is now bordered by a sharply defined periphery.

Since this enlargement distributes the same amount of light over a much greater space, the picture thus produced will be short of light, and this loss naturally will increase with the strength of the eyepiece. Hence a certain limit is set to ocular magnifying, and a power greater than twelve should not be used, at least not with a *Huyghen* eyepiece.

Eyepieces are generally numbered in Roman or Arabic figures, and naturally a corresponding decrease in size of the lenses and in length of the eyepiece is noted in proportion with the decrease in strength.

ILLUMINATION

Illumination by Mirror.

So far we have assumed that our object itself sends out rays of light spontaneously, which in reality is not the case. We therefore illuminate our specimen artificially in order to enable it to radiate light. This illumination may be brought about in two ways: either from above when the section is impermeable to light, or from below through the opening in the stage. The former method, using **reflected light,**[1] is of little interest to us, as most all examina-

[1] In this method the *Bull's eye condenser,* so-called, is used.—(The Translator.)

tions are made with the aid of the latter, the ***transmitted light,*** method. Illumination here takes place from below by means of the movable mirror, which, as stated before, is plane on one side, concave on the other.

Assuming that we are using the ***plane*** mirror, a luminous cloud furnishing the source of light, the parallel rays emanating from the latter are caught by our mirror and reflected into the specimen. Each point of the latter thus sends its slightly diverging bundle of rays into the objective. As each bundle must needs completely fill out the angular aperture of the objective, in order to obtain good illumination, we can use this plane mirror only with objectives of a small aperture, to wit, weak objectives. When using stronger objectives, we must have bundles of light, which have a greater divergence, and these will only be furnished by a ***concave*** mirror, which causes the parallel rays to converge and which is so adjusted as to focus in the objective. Rays of about 40° divergence can be obtained from this mirror, which is absolutely sufficient for medium strength objectives.

Illuminating Apparatus.
Condensers.

For high power enlargements still more divergent bundles will be required, in order to exhaust the high apertures of the objective, and it is in these cases that an extra system of lenses must be interposed between mirror and specimen, commonly known as the ***Abbé condenser.***

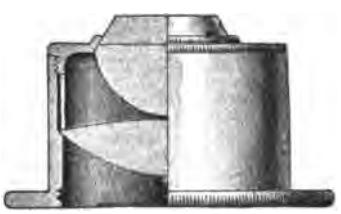


FIG. 14.
Abbé's Condenser.

This illuminating apparatus is composed of two lenses (Fig. 14), which are snugly inserted in a metal case. The entire system can be adjusted higher or lower and even everted entirely by a screw, seen in Fig. 8. The upper of the two lenses is more than a hemisphere, while the lower one is a biconvex lens. The two lenses combine to form a strong collecting system, rendering the rays, coming from the plane mirror, strongly convergent, so that they emerge strongly divergent. Our condenser has an aperture of 1.20, furnishing bundles of rays of 104° divergence. The minute loss of luminosity, caused by the air between condenser and slide, may be obviated by a drop of immersion oil so applied as to fill out this air-space.

Since, in most cases, the entire amount of light furnished by mirror or condenser is not required nor even desirable, the cone of light is regulated by an adjustable device, named the ***diaphragm,*** which is interposed between mirror and condenser and is capable of narrowing the cone of light in its course from the mirror to the condenser to a minimum.

The Iris Diaphragm.

We utilize for this purpose the so-called ***Iris diaphragm,*** which is found closely below the lenses of the condenser, being attached to the metal case containing the latter. Such an Iris diaphragm consists of a number (12—18) of thin small metal plates, each resembling a sickle or crescent with a short handle, the point being obliquely cut off. These are arranged in a thin metal drum in such a manner, that they cover one another almost entirely when the diaphragm

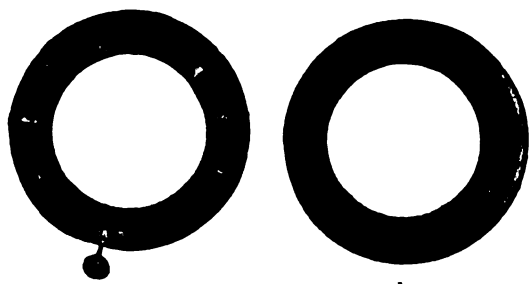
is open (Fig. 15 *a*), leaving only their ends visible, thus forming a broad ring. Each crescent forms a lever, its fixed point being located in the handle. A small rod is found on the distal end of each crescent, which plays in a corresponding minute moat. These depressions are arranged in a radiating manner on a metal ring, which is provided with a lever, the ***diaphragm lever*** (Figs. 8 and 16 *a*). If the lever be turned to the right, all the crescents advance evenly toward the middle, thus bounding a polygon, having as many

a *b*

FIG. 15.

Iris Diaphragm. The Cap and Lever have been Removed. *a*, Completely Open; *b*, Closed.

sides as there are crescents and being nearly circular (Fig. 15 *b*). The more the lever is turned to the right, the smaller will be the diaphragmatic opening,



a *b*

FIG. 16.

Iris Diaphragm. *a*, Lever-Ring; *b*, Cap.

the minimum being one millimetre diameter. This well-devised apparatus thus enables us to regulate the light ad libitum.

Under certain circumstances, especially when using low-power lenses, it is of advantage to be able to switch out the condenser entirely. The place of the diaphragm will then be taken by the so-called ***cylinder diaphragm,*** which can be fitted into the opening in the stage and which is manufactured with variously sized openings. The light can thus be regulated by the various sizes of cylinder diaphragm used, but aside from this the amount of illumination can also be reduced by lowering the same cylinder to a desirable level.

Source of Light.

The best source of light for our microscope is a light cloud, a blue sky being unfavorable, while direct sunlight must be absolutely avoided. Of the

artificial lights the most serviceable and convenient is the gas-mantle burner. It is a good plan to place a reflector behind the lamp and to interpose a sheet of tissue-paper on a wooden frame between lamp and mirror, at least when low power is used.

THE MICROMETER-SCREW

Micrometer-Screw.

Lastly, it behooves us to speak of the micrometer-screw, which constitutes by far the most important mechanical part of the microscope and requires the most careful manipulation. Looking at Fig. 17 we will easily understand its construction. Here we find the upper end of the pillar, the hoodlike covering of the micrometer-screw having been removed. As previously mentioned, the pillar above the stage takes on a prismatic shape and is enclosed by the carrier. This prism ends in a cylindro-conical knob, projecting from the carrier, as shown in our figure. This plug as well as the prism are hollow, the former being slit in addition on its top. Through this slit a bridge is inserted, which is screwed to the top of the carrier. A strong spiral spring, lodged in the hollow of prism and plug, exerts pressure against this bridge, thus pressing the bridge and the intimately connected carrier upward. This pressure is counteracted by the micrometer-screw proper, a short, strong steel screw of perfect execution, its freely movable tip pressing against a depression in the bridge. Turning the screw to the right we will compress the spring and thus lower the tube-carrier. In this manner the carrier may be lowered until its lower end approximates the enlargement of the pillar found just above the stage (Fig. 8), in other words, until the entire prism is covered. Turning to the left, the carrier, pushed upward by the spring, is raised; this can be done until the bridge touches the ceiling of the slit, after which the carrier will cease to rise, and if the turning is continued, the micrometer-screw will simply be taken out.

FIG. 17.
Micrometer-Screw.

We may add that the different makes of micrometer-screws vary essentially in their construction.

SOME RULES FOR THE USE OF THE MICROSCOPE

The Procuring of a Microscope.

In the manufacture of microscopes Germany has taken first place for years past, and such renowned firms as Zeiss, in Jena, Leitz and Seibert, in Wetzlar, Winkel, in Goettingen, Voigtländer, in Brunswick, and Reichert, in Vienna,

furnish excellent instruments, which can be said to be equally efficient in their essentials. It is therefore partly a matter of personal preference or, perhaps, partly a pecuniary question, which make should be selected by the prospective buyer.

An instrument answering all the requirements of a beginner may be bought nowadays at a price of 300 to 400 marks (75 to 100 dollars), and such an instrument will, moreover, prove satisfactory for the work of the general practitioner.

The objectives should be thoroughly tested as to their efficiency, and their selection should best be left to an expert. One should also convince himself of the faultless construction of the micrometer-screw by sharply focusing at different heights.

The Care of the Microscope.

It scarcely needs mention that an instrument of such minute precision as the microscope requires the most scrupulous care and delicate manipulation. It should be protected from dust by replacing it in the case after use, or, still better, by keeping it under a bell-glass jar. In transporting the microscope one should make it a rule to always grasp the foot and not the tube-carrier, as is generally practised.

The Cleaning of the Objective.

The objectives require the most rigid care. If the front lens is soiled, the entire objective should be unscrewed from its nosepiece and the lens cleaned with a very soft cloth, preferably a well-laundered handkerchief. We hesitate to recommend the chamois leather, which, although very useful, is soon soiled. If the lens cannot be cleaned satisfactorily with the dry cloth, the latter should be moistened with alcohol. If the front lens is clean and still the objective does not furnish a clear picture, it will be best to have the optician look it over. Under no circumstances should the beginner attempt to disarticulate the component lenses with a view of cleaning the same separately. It goes without saying, that the objective, or more particularly the front lens, must be protected from all acids, alkalies or such solutions as are solvents of fats and resins.

The microscope should never remain without an eyepiece or without proper apposition of the nosepiece, as otherwise dust will accumulate on the interior, making it difficult to keep the lenses clean.

The Focussing of a Field.

It seems advisable to say a few words regarding the focussing of a slide, a process which often proves quite difficult for the beginner. We found that each objective has its own fixed object distance, and that the latter decreases with the increasing strength of the lens. When handling a high-power lens it may thus easily happen that the front lens comes in too intimate relation with the cover-glass. One of two things, or both, may happen. Either the cover-glass will be crushed and the specimen ruined or, what is more serious, the front lens may be loosened from its setting. This might especially happen when using an immersion lens, where, as we have found, the metal setting of the minute hemisphere lies attached only to a very small ring.

It is an excellent plan for the beginner to establish the rule to examine first with a low-power lens. Any particular part of the field should then be moved to the middle. It will then only be necessary to shift the nosepiece and to use the fine adjustment, since the length of the objectives has been graduated in a manner so as to make the coarse adjustment for all objectives the same.

Low-power objectives, e.g., as No. 3, have such a great distance as to render the focussing safe. If trouble should be encountered here also, it is best to proceed by raising the barrel rather than lowering it. The front lens is first brought down close to the cover-glass, then, while looking in the microscope, the barrel is raised by means of the coarse adjustment screw, until the picture appears.

When the oil-immersion lens is to be used, that portion of the specimen is brought to the middle of the field, focussing first with a low-power and then with a medium lens, the barrel is then raised and the nosepiece turned. A small drop of immersion oil is placed in the middle of the cover-glass and the barrel lowered until the front lens comes in contact with the drop of oil. If a small drop of oil has been used, the image will now be visible, though indistinct, and all that remains necessary is the use of the micrometer-screw. When the examination has been completed, the front lens and cover-glass are cleansed with a cloth moistened with concentrated alcohol.

During the entire examination the hand should be kept on the micrometer-screw, the latter following each glance of the observer and making it possible for the eye to penetrate the various depths of the specimen; hence its great importance to our instrument. The micrometer-screw should never be used for coarse adjustment, and one should always observe that the prism, mentioned before, is visible at all times. If any resistance is felt when focussing, the cause of same should be found before any further adjusting is attempted. The micrometer-screw will be found lowered to its limit or the front lens has touched the cover-glass. If the turning of the micrometer-screw has no effect on the focus, it will be found to have passed its upper limit. Too great a part of the prism will be visible in this case, and the fine adjustment must be lowered until the correct distance is attained.

General Microtechnique

Definition and Purport of Microtechnique.

By microtechnique we understand the totality of all those methods which serve to produce a microscopic slide. This technique depends on the one hand on the peculiarities of our microscope, on the other it is governed by the properties of the specimen about to be examined. We will best make ourselves clear by citing a simple practical example.

Let us suppose that we are to prepare a specimen of the human kidney. First we are confronted with the proposition of making a ***section.*** This section must not be made in a coarse, superficial manner, but must be so perfected that a transparent preparation is obtained, since we want to examine our slide with transmitted illumination. We can either make thin sections with the cutting instruments designed for this purpose, or we may separate minute particles by means of scissors, and then still reduce their size by teasing with teasing needles. We now put our specimen on a thin glass plate ***(the slide),*** and cover it with a still thinner small glass plate ***(the cover-glass).*** When using high-power objectives, the latter must only be a fraction of a millimetre in thickness, on account of the small object distance.

As all these manipulations require time and sometimes a good deal of time, our specimen will in the meantime suffer changes. In all animal organism great changes take place soon after death, which alter its original structure to a high degree, and which occur all the sooner if the organism has been finely divided and separated from its component structures. The question thus arises, how may we delay these changes or, if possible, prevent them, i.e., ***fix*** our specimen. But supposing that we have made fine transparent sections of a kidney and fixed them in their original structure, we will meet with still another difficulty. The various tissues comprising the specimen, e.g., epithelium, connective tissue, nerve tissue, etc., differ so little in their optic properties, that their differentiation will be extremely difficult to the untrained eye, if not impossible. The examination will therefore be materially facilitated by subjecting the preparation to a dyeing process, i.e., by ***staining*** them.

We may thus divide microtechnical methods into three great groups, namely:

1. ***The Method of Preservation.***
2. ***The Method of Making Sections.***
3. ***The Method of Staining.***

For all these methods scores of more or less complicated instruments have been devised during the last fifty years, and a host of chemicals, reagents and

dyes have been recommended. We shall now acquaint ourselves with these, at least as far as their practical value for us is concerned.

METHODS OF PRESERVATION

Observation of the Living Object.

The preparation of a specimen is not always as unfavorable and difficult as in the case of the human kidney. Going down the scale of organisms, we find thousands of forms, which are so minute and transparent that we can put them on our slide without further preparation and keep them there alive under observation, for hours and even days. But also higher organisms, even human, can be cut and their sections kept alive and studied for some time. For instance, it is not very difficult to observe living human blood or spermatic fluid under the microscope.

Indifferent Liquid Media.

The great importance in these observations is to examine the object under conditions as nearly normal and natural as is available. Just as we would examine infusoria, obtained from a sweet-water aquarium, in this same medium, we likewise proceed with human blood corpuscles, i.e., we do not put them in water, where they would instantly undergo hæmolysis, but select as a medium either blood serum or at least a liquid of similar properties, i.e., an ***isotonic*** fluid. Of course, care must be taken that this fluid does not evaporate and thus become concentrated. If, on the other hand, a ***hypotonic*** fluid should be selected, for example, distilled water, osmosis would take place, the liquid entering the corpusles and distending them. If a ***hypertonic*** solution was used the result would be a loss of fluid from the corpuscles with consequent shrinkage.

The most satisfactory results for mammalian tissues are obtained with ***blood serum,*** which may be secured by centrifuging cooled blood, or with ***amniotic fluid,*** which may easily be saved from the gravid uterus of the cow or pig. The ***aqueous humor*** from the anterior chamber of the eyes of larger animals may also be utilized for this purpose, and can be procured by puncture with a *Pravaz* hypodermic needle.

Of the artificial indifferent media the ***normal*** or ***physiologic saline solution*** is the best known. It should contain 0.9% of table salt for examination of mammalian or human tissues. A still better solution is ***Ringer's fluid,*** containing NaCl, 8.0 gms., KCl, 0.2 gm., $NaHCO_3$, 0.2 gm., and $CaCl_2$, 0.2 gm. in 1,000 cm^3 of distilled water.

Granted that the study of living organism is of great importance in the physiological and biological research into the animal body, the fact remains that morphology deals to a greater extent with specimens which have previously been killed and preserved in the proper manner.

Significance of Fixation.

Naturally the first prerequisite is, that the tissues are preserved as nearly as possible in their original structure and relation. The object is, therefore,

to fix the structure, and the methods and reagents employed for this purpose have been termed ***fixation,*** and ***fixing solutions*** or ***fixatives.*** If the object is thus killed in a state truly resembling that of life, it can easily be preserved for an unlimited time.

In order to familiarize ourselves with the fixation of animal tissue, it will be a good plan to branch off for a short excursion into physiologic chemistry. The cells and the intercellular substance composing the body tissue, consist chiefly of water, minerals, carbohydrates, fats and albumins. The latter are for us the most important of the constituents of tissues, high-molecular, very complicated compounds, which are partly neutral, partly acid and partly basic in reaction, being soluble in water, weak alkalies or acids. As far as microtechnique is concerned, their most important property is that they are made insoluble ***(coagulate),*** when treated with certain physical or chemical agents. One of the best known and most common physical coagulating agents for albumin is ***heat.*** If an albumin solution is heated to nearly 100°, all the albumin becomes coagulated, provided the solution be slightly acidified. The albumin now has become insoluble in its former solvent, it is denatured. If a ***mineral acid*** or certain organic acids are added to an albumin solution, coagulation will result with the production of an acid albumin, which, however, is soluble in the diluted acid. Another important group of substances coagulating albumin consists of the ***salts*** of the ***heavy metals.*** These change the albumin into metal albuminates, which are insoluble in water, i.e., loose compounds of metal oxides with albumin. ***Alcohol*** also deserves mention as a coagulating agent. If an albuminous solution is treated with alcohol, the albumin coagulates, again becoming soluble when water is added. In this case, therefore, it is not denatured. If, on the other hand, the alcohol is allowed to act for a longer time, it also will render the albumin insoluble. ***Formalin,*** a solution of formaldehyde in water, of late more widely known in science and practice, forms methylene compounds with most albumins, which compounds are insoluble in water.

This property of albuminous substances is of great interest in microscopy, enabling us to fix the inconstant tissue picture by coagulating the otherwise soluble albumins.

Thus, fixing means for us nothing more than the coagulation of albuminous bodies, wherefore every good fixing agent must be a good coagulant of albumins. But again, an efficient albumin coagulator need not be a good fixing reagent; e.g., tannic acid, while causing albumin to coagulate readily, is totally useless as a fixative.

Practical experience during the last fifty years has demonstrated to us many methods and reagents, which might be used for fixation, but we have also learned that it is often of advantage not to depend upon a single reagent for fixation, but to combine the good properties of a number of them; thus alcohol or metal salt solutions are used in conjunction with an acid, commonly acetic acid, each of which would yield poor results if used alone.

General Rules for Fixation.

Before proceeding to the special discussion of the various fixatives, it might be well to dwell on some general and practically important points. If a

true reproduction of our preparation is desired, it follows that we must fix our specimen in the living state, which proves to be quite a difficult task. When dealing with animals, the living object is more often available and the specimen can be taken directly after the animal has been killed and may immediately be put in the fixing solution. With human material the conditions are rarely so favorable.

Anæsthetizing and Killing of the Animals.

Most commonly ***chloroform*** is used in the killing of animals. Smaller animals, up to the size of a small dog, should best be put under an adequately large glass globe or bell glass. A tuft of cotton, saturated in chloroform, is introduced, the animal carefully watched and the glass held fast, since many animals, foremost among them the cat, exhibit a state of tremendous excitement. When the animal has become motionless and reactions have ceased, it is attached to an operating board; the thorax is quickly opened and death is brought on by removing or opening the heart. Larger dogs better receive an initial dose of ***morphine*** (6 cm^3 of a 1% solution of morphine muriate) subcutaneously and, after half an hour has elapsed, will easily succumb to chloroform administered on a cloth. A very convenient and humane method of killing smaller animals is the administration of ***carbonic acid*** by means of the so-called carbonic acid bomb, whence the gas is led through a tube into the bell glass. In small animals like frogs, mice, rats, etc., death is generally induced by ***cutting off their heads*** with strong scissors. When using a frog, it is best to ***destroy the spinal cord*** in addition, employing a medium sized probe for this purpose. Another very useful but dangerous agent is a ***2% alcoholic solution*** of ***hydrocyanic acid,*** which is kept in stock in most drug stores. A few drops, injected subcutaneously, kill even larger animals almost instantaneously.

Specimens from the Human Body.

In the examination of human material much time is often lost before it reaches us, unless we are dealing with an operative case or, perhaps, an autopsy. Material taken two to three days *post mortem* is utterly useless for our purpose. The maximum time which may elapse between death and the taking of a section is twelve hours. A very efficient and easily obtainable material, which serves well for most purposes, is the body of a child during the first few weeks after birth.

Size and Treatment of the Specimen.

Since the ***penetrating power*** of most of our fixing agents is moderate, it is best to use small or at least thin pieces, unless other reasons make this impracticable. A section of 1 cm thickness might be considered as very thick.

The selected section should not be handled unnecessarily with instruments, and, in order to avoid any possible crushing, the section should only be made with sharp instruments, scissors or razor.

The ***amount*** of ***fixing solution*** should be plentiful, the volume being at least twenty times that of the object to be fixed.

To allow free access of the fixative from all sides, the specimen is best suspended in the solution, or else the floor of the jar should be covered with glass wool, cotton or several layers of tissue-paper.

Duration of Fixation.

The ***duration*** of the fixing process naturally depends on the size of the object and the nature of the fixing agent. A general rule may be adopted that the process should not last longer than is necessary for complete saturation of the specimen. The speediest result is obtained from acids; alcohol and formalin taking slightly longer, while the metal salts work slowest of all; e.g., after acting four hours, a 5% nitric acid solution[1] will have penetrated 6 to 7 mm, 10% formalin, 5 to 6 mm; 96% alcohol 3 to 4 mm, 0.3% platinum chloride only 0.5 to 1 mm. Only a few fixing solutions can act on a specimen for an indefinite period without injuring it, i.e., can also serve as a preservative. Of these we may mention primarily 5 to 10% formalin, in which a specimen can remain almost unchanged for an indefinite time. To a certain extent this can be said of alcohol, which should be used 70 to 80% strong. All other agents change the specimens under prolonged use; they partly render them hard and brittle, or partly deposit themselves in crystal form, thus rendering the staining difficult, if not impossible.

FIG. 18.

Jar for Washing of Fixed Specimens.

After-Treatment.

We find, therefore, that after most fixation methods we must seek to ***extract*** the fixing solution after it has done its work. This is generally done by means of a ***wash-glass*** (Fig. 18), which is suspended on the knee-shaped limb in a water reservoir, fed from the faucet. Our specimen thus lies in a continuous current and within a few to twenty-four hours the fixative will be entirely washed out. Some of our solutions cannot be treated that way, and foremost among them are the acids. Here we can use 70% alcohol, which must be renewed frequently, or 5% formalin.

We will now proceed to discuss a number of the most important fixing solutions which are of interest to us.

1. ***Nitric acid,*** HNO_3, when pure and concentrated, is a colorless liquid of 1.54 specific gravity; when exposed to air, it soon assumes a yellow color, due to the formation of nitrous acid. The commercial concentrated nitric acid, which is generally used, contains 68% HNO_3 and has a specific gravity of 1.41. The C. P. nitric acid, obtained at druggists, contains only 25% of HNO_3, having a specific gravity of 1.15. When nitric acid is mixed with alcohol, the main compounds formed are: Nitric acid-ethyl ether ($C_2H_5O.NO_2$), nitrous acid-ethyl ether ($C_2H_5.ONO$) and acetaldehyde ($CH_3.CHO$). It must be borne in mind that the ethers of nitric acid are quite explosive compounds under certain conditions.

[1] When speaking of any per cent. solution, we naturally always mean that per cent. solution in distilled water.

Nitric acid itself, or its compounds, is one of our most valuable fixing agents, and especially for certain organs, e.g., the retina, it cannot be excelled. It is mostly used in a 7.5% solution, i.e., 7.5 cm^3 nitric acid of 1.41 specific gravity and 92.5 cm^3 of water.[1] This agent penetrates the tissues very well, indeed better than any other fixative. Nitric acid must not be allowed to act too long on tissues, as it will otherwise become a solvent of certain elements of the cellular tissue—five to six hours may be considered the maximum, although ordinarily three to four hours will suffice. If the object should be removed from the acid and directly placed in water, a swelling of the connective tissue will take place, wherefore the acid must first be taken out with other solvents, e.g., 70% alcohol, 5% solution of *Glauber* salt, or 5 to 10% formalin.

2. ***Acetic acid,*** $CH_3.COOH$, a colorless liquid of intensely pungent odor. Specific gravity, 1.05. It crystallizes at about 70° F., whence it is often called ice-vinegar. The boiling-point is approximately 298°, and the acid burns with a blue flame. It must be kept in a well-corked bottle, since it is extremely hygroscopic when exposed to air.

By itself this acid makes a poor fixative, but as an ingredient of other reagents it plays an important rôle by virtue of the rapidity with which it diffuses through the tissues, rendering them acid and thus preparing them for the action of the actual fixing agent. The vapors of acetic acid are also used for fixation in certain instances.

3. ***Trichlor-acetic acid,*** CCl_3—COOH, a compound in crystal form, dissolving when exposed to air and having caustic properties.

A 5% solution is quite a useful fixative, acting much slower than nitric acid, hence a specimen must be allowed to remain up to ten or even twelve hours. The acid is easily washed out with 95% alcohol.

4. ***Picric acid,*** $C_6H_2(NO_2)_3.OH$, a yellow crystalline powder, slightly soluble in water (1.1%), more readily in alcohol, forming a bright yellow solution.

Picric acid solutions, which are strongly poisonous, do not readily penetrate the tissues and *per se* are of little significance for our purpose. On the other hand, picric acid becomes a potent factor when forming a constituent of a solution, and is especially important as a stain.

5. ***Chromic acid,*** $Cr\,O_3$, a reddish brown crystalline compound, soluble in damp air. It is strongly caustic and is reduced by alcohol into brown chrome hyperoxide, respectively green chrome oxide. Chromic acid solutions should not be filtered through paper.

The acid is used in a 0.25 to 1% solution, but rarely. Its penetrating power is low, although greater than that of picric acid. The object is usually left in the solution for twenty-four hours, after which it is washed in running water for an equal length of time. Any precipitate which may have formed in the tissues can be removed by treating the section with a 5% solution of potassium cyanide. An objection to the use of chromic acid lies in the fact that specimens so treated become brittle and are stained with difficulty.

6. ***Osmium tetroxid,*** $Os\,O_4$—incorrectly named osmic acid, is manu-

[1] When speaking of water we invariably mean distilled water; if well or tap water should be used, it will be specified as such.

factured in the form of slightly yellow crystals, which are put up in small glass tubes of 0.5 or 1.0 gr, on account of its great diffusibility.[1] Its vapors are very irritating to the mucous membranes. After thoroughly cleansing the tubes, they are filed on one side and opened by touching them with a glass rod heated to white heat. In order to waste nothing, the entire tube with contents is put in an absolutely clean bottle and corked with a tightly fitting glass stopper. The necessary amount of water can now be added—3 to 4% will make a saturated solution. The solution must be protected from dust.

Osmic acid has attained great importance in microtechnique owing to its properties as a fixing agent. Although only moderately able to coagulate albumin, it makes an excellent fixative for the cell, particularly the cell body. It is used in 0.5 to 2% solution, or in the form of vapor. Thin objects are exposed to the vapors of a 20% solution for a few minutes. Osmic acid solutions penetrate the tissues only slowly, although not as slowly as is commonly stated. Two to three millimetres are saturated in a few hours. The staining of osmic acid preparations is not so unsatisfactory, as is often supposed, provided that the acid has been thoroughly washed out in water for twenty-four hours, and that it has not been treated with alcohol afterward. To preserve such specimens 5% formalin is to be recommended.

Another very important advantage of osmic acid lies in the fact that it is reduced to different degrees by animal tissues, thereby furnishing a finely shaded picture. It is most strongly reduced by fat and some deutoplasmatic bodies, which in consequence appear totally black, while the remainder of the picture shows gray and grayish brown hues. A secondary staining process can therefore often be dispensed with.

To decolorize osmic acid preparations, the sections are best treated with an alcoholic solution of hydrogen peroxide (one part of the commercial peroxide to 5—6 parts of 70% alcohol).

7. ***Chrome-Osmio-Acetic Acid.***—A mixture of 15 cm^3 of chromic acid (1%), 4 cm^3 of osmic acid (2%), and 1 cm^3 of acetic acid, which has received the name of *Flemming's* solution, has a wide range of usefulness in microtechnique, and constitutes one of our best fixing solutions. In rapidity of penetration it excels chromic acid as well as osmic acid. The staining properties of specimens so treated are excellent, inasmuch as no alcohol is used for after-treatment. The section is usually fixed for twenty-four hours, but might be left in the solution for several days without any harm resulting therefrom. Careful washing in running water for twenty-four hours after completion of fixation is indispensable.

8. ***Potassium Bichromate,*** $K_2 Cr_2 O_7$.—Red trigonal crystals, 10% soluble in water, giving a faintly acid reaction. *Per se* potassium bichromate is an inadequate fixing agent, since it is not able to fix those albuminous parts of tissues, which are most valuable to us. It penetrates slowly but steadily. If one desires to use this reagent, it must be acidified first, and this can be accomplished with acetic acid. To a 3% solution add 5% of acetic acid and fix for

[1] Osmic acid is at the present time a very expensive reagent, selling at about $2.00–$2.25 per gram.

twenty-four to forty-eight hours, after which the section must be washed in running water for the same length of time.

9. ***Mueller's fluid,*** a reagent formerly used extensively, is to this date a very efficient fixing solution. To prepare it, 2.5 gms of potassium bichromate and 1 gm of sodium sulphate are dissolved in 100 cm^3 of water. It is especially adapted for the preparation of the central nervous system for subsequent staining of its nerve fibres. But even as a fixing agent solely *Mueller's* solution gives better results than the bichromate solution alone, which is probably due to a liberation of small amounts of chromic acid by the addition of the *Glauber* salt. The solution is allowed to act for days, weeks and even months, but it must be changed as soon as it becomes turbid. Entire human brains used to be treated in this fashion for months, a procedure which has today been abandoned as coarse and primitive. After fixation the running water bath is essential. Staining properties of *Mueller* preparations are good for the usual carmine and hæmatoxylin solutions. In some cases the preparation with *Mueller's* fluid is even a prerequisite. The chrome salt present acts as a corrosive.

10. ***Potassium-Bichromate-Osmic Acid.***—Of the other potassium bichromate mixtures the only one of interest to us is the potassium-bichromate-osmic acid, usually designated as *Altmann's* mixture. It consists of equal parts of 2.5% potassium bichromate solution and 2% osmic acid solution. Small pieces are fixed in it for twenty-four hours and are washed equally long in running water.

11. ***Corrosive Sublimate,*** $HgCl_2$, occurs in white crystals, soluble in water to the extent of 7%, 25% in alcohol, giving an acid reaction. The solution is not stable and easily breaks up on exposure to light with the production of calomel. More stable compounds can be produced by using normal saline solution instead of distilled water, the former giving us a neutral bichloride solution by the formation of a double salt compound, chloride of mercury-chloride of sodium. If the tincture of iodine is added to a sublimate solution, a decolorization of the latter with the production of mercury iodine takes place.

Corrosive sublimate energetically coagulates albumin. Its aqueous solutions, which are acid in reaction, reduce the genuine albumins to insoluble albuminates. Albuminates furnished by the mercury chloride-sodium chloride solution become insoluble only on addition of an acid. The penetrating power of corrosive sublimate is medium, pieces of 5 mm thickness being completely fixed within four to five hours; 2.5 to 5% watery solutions are used, and it is to be recommended to make your solution fresh just shortly before using it. Such recent solutions give decidedly more constant results as do older table salt-sublimate solutions. The solution can be used with or without additional acidification.

12. ***Corrosive Sublimate with Acids.***—For acidifying, various acids can be utilized. One to two per cent. acetic, 7.5% nitric or 10% trichloracetic acid all work well. Specimens should not remain longer than necessary in neither the pure sublimate nor the acidified solutions. For small sections one hour should suffice; larger specimens may remain six to eight hours, but twelve hours should never be exceeded, as the shrinking caused by this solution increases with the time consumed in fixing. Washing of the section in

running water for twelve to twenty-four hours should always be practised with sublimate as well as sublimate-acetic acid solutions, while the remaining acid mixture methods are best followed by strong alcohol, to avoid a swelling of the connective tissues. Material for frozen sections should be washed and placed in 5% formalin. Corrosive sublimate always causes coagula in the connective tissues, which cannot be removed even by the most scrupulous washing. They can be removed either in the section or in the bulk. The former is done in frozen sections, where the coagula are dissolved by placing the section overnight in a 5% solution of sodium sulphate and afterward washing it, changing the water frequently. When dealing with specimens which are to be embedded in paraffin, the coagula are best removed by the use of 70 or 80% alcohol, to which has been added enough of the tincture of iodine to give it a good brown color. The iodide of mercury is thus formed, which is soluble in alcohol, giving us a colorless solution. If, therefore, a zone of colorless alcohol is found around the specimen on the following morning, the process must be repeated, until no more decolorization is observed.

Corrosive sublimate, despite the fact that of late it has lost some of its reputation, still remains one of our very best fixing agents; objects prepared by its use, aside from excellent preservation of structure, show great staining properties.

13. ***Corrosive Sublimate - Mueller's Fluid - Acetic Acid Combination,*** known as *Zenker's* fluid.—To 100 cm^3 of hot *Mueller's* fluid 5 gms of bichloride of mercury are added, and just before use this solution is acidified with 3 cm^3 of acetic acid. Duration of action and after-treatment are the same as with corrosive sublimate, but the staining properties of the latter are far superior.

14. ***Corrosive Sublimate - Chrome - Osmium - Acetic Acid.***—To 100 cm^3 of a 1% bichloride solution we add 10 cm^3 of a 10% chromic acid solution, 5 cm^3 of a 2% osmic acid solution and 2 cm^3 of acetic acid. Duration of fixation is twenty-four hours, to be followed by washing in running water for an equal length of time.

15. ***Platinum Chloride.***—The product sold under this name is really platinum chloride—hydrochloric acid, $Pt\,Cl_4 + 2\,H\,Cl + 6\,H_2O$; it forms reddish brown crystalline masses, which are highly soluble in water, making a strongly acid solution. Owing to the fact that this compound is acted on by light, it is best to keep it in well-corked brown bottles.

16. ***Platinum Chloride-Osmium-Acetic Acid.*** — Platinum chloride acts well on albumin and therefore is an excellent fixing agent; however, it possesses little penetrating power. It is generally made use of in the form of platinum chloride-osmium-acetic acid or *Hermann's* fluid, which consists of 15 cm^3 of 1% platinum chloride, 4 cm^3 of 2% osmic acid, and 1 cm^3 of acetic acid. In its action it is very similar to *Fleming's* solution. Specimens are fixed from twenty-four hours to several days, and then washed for twenty-four hours. The staining properties are not as good as they are obtained from *Fleming's* solution.

17. ***Alcohol.***—Ordinary ethyl alcohol, $C_2\,H_5\,OH$, when free from water, is an excellent fixative of albumin and, if allowed to act long enough, furnishes durable denaturation products. At the same time it dehydrates the specimen,

so that the section may be rendered free from water by the repeated use of alcohol. Alcohol, when judiciously used, is a very good and, as we will see later, a very convenient fixative. As regards its penetration alcohol takes a medium rank, ranging somewhere after potassium dichromate in speed. The staining properties are good, although they are generally overestimated. A good fixation can only be obtained from absolute or at least very strong alcohol, and here again we find a disadvantage in that the water is extracted too quickly from the section, so that delicate moist tissues are apt to shrink enormously.

The so-called absolute commercial alcohol contains 99.4—99.6% of pure alcohol, which is absolutely sufficient for our purpose. It must be kept in well-corked bottles, since it takes up water assiduously from the atmospheric moisture.

Large quantities should be used and the sections suspended freely; in that fashion it will penetrate 8—10 mm within twelve hours. If a specimen is left for several days, it becomes very hard; it is well, therefore, to transfer it to 70—80% alcohol for the purpose of preserving a section. After a time the staining property of such preparations is materially decreased.

18. ***Alcohol Acetic Acid.***—Combinations with alcohol are innumerable, all designed for counteracting its shrinking property. The most important adjuvant is acetic acid. One part of the latter is added to 4 parts of alcohol (absolute), the mixture is allowed to act for twenty minutes, after which the section is transferred to a mixture of 1 part of the acid to 8 parts of alcohol, and hence to pure alcohol, which must be renewed after a few hours. Of the remaining alcohol-acetic acid mixtures *Carnoy's* is the best known. It consists of 60 parts of absolute alcohol, 30 parts of chloroform and 10 parts of acetic acid. It penetrates well and furnishes a good nuclear and plasma fixation. Fifteen to twenty minutes for ordinary, an hour for larger pieces, suffices for fixation, after which the object is transferred to absolute alcohol, which must be changed several times within the next few hours.

19. ***Formalin.***—Formalin is a 40% solution of formaldehyde, H.COH, in water; it is a colorless fluid with a pungent odor, not only irritating mucous membranes, but even producing an itching erythema on susceptible skin. When fresh, formalin is neutral in reaction, but it gradually becomes acid, due to the liberation of formic acid.

As previously mentioned, formalin can coagulate a large number of albuminous bodies. It possesses a good penetrating power and in no way diminishes the staining properties.

Formalin is used in 10% solution, and specimens can be kept in this fluid for an indefinite period, so that this solution or, perhaps better, a 5% strength, makes a good preserving agent as well. After fixation specimens can be directly transferred to diluted alcohol, since it is not necessary to wash out the formalin.

While formalin cannot be considered among the very best, it certainly is a good fixing agent, and for certain tissues, e.g., muscle, it excels all others. When properly used it does well in almost all instances.

20. ***Formalin-Mueller's Fluid.***—Of the various compounds of formalin *Mueller's* fluid with formalin, in the proportion of 9 : 1, is the best known.

By the addition of formalin the fixing property of *Mueller's* fluid is materially increased. When the mixture becomes milky it must be changed. Specimens should be fixed for twenty-four to forty-eight hours, washed in water and preserved in 5% formalin.

21. ***Formalin-Alcohol.***—Combinations of alcohol with formalin are also used extensively. Absolute alcohol, containing 10% of formalin, will furnish a mixture which shrinks the tissues less than does alcohol alone. Twenty-four hours are allowed for fixation, and according to what after-treatment is desirable, the specimen is transferred to absolute or diluted (70%) alcohol.

PREPARATION OF SECTIONS

The methods of making microscopic sections, which we will now deal with, all have in common the object of reducing the voluminous organs of the body into small, thin, transparent sections, which allow the rays of light to pass through them, thus giving us a specimen which is adapted to microscopic examination. This end may be attained in many different ways. Specimens can be teased, crushed, chopped, shaken, cut, etc. All these methods have two objects in view: either it is desired to preserve the relationship of constituent parts of a tissue, or to dissolve such relation by dissociating or isolating the component factors. The former purpose is best accomplished by the ***cutting method,*** which enables us to make sections of any desired thickness, either of the entire organ or of smaller parts of same. The various cutting methods are by far the most important and therefore the most thoroughly understood means of preparing a specimen. Aside from these, the ***dissociation methods*** enjoy some of our interest by making it possible for us to study the individual forms of the tissue elements. The ***chop-method*** takes a position midway between cutting and dissociation methods, and may be used with advantage in some cases.

THE DISSOCIATION METHODS

Most tissues resist the mechanical dissociation of their component parts; e.g., it is impossible to separate a muscle fibre from the tendon fibre to which it is attached, both being united by a cement substance. The same is true of many other tissues, so that our first aim will be to dissolve this cement chemically, after which the mechanical isolation can take place without difficulty.

To accomplish the former, so-called isolation- or maceration-agents are used, of which we have quite a large selection. We will only mention those which are most important for our purpose.

Natural (Spontaneous) Maceration After Death.—After tissue death the tissue juices furnish a medium, which, for instance, dissolves the cement substance, gluing together the epithelial tissue. For this reason we find that in objects which are fixed only after the elapse of some time after death the epithelial lining has been stripped from the underlying tissue.

Alcohol.—A fine isolation agent for epithelium is found in dilute alcohol, 1 part of 90% alcohol to 2 parts of water. Epithelial tissues may easily be macerated after twenty-four hours.

Acetic Acid Vapors.—Better still are acetic acid vapors. The mucous membrane in question is spread on a wax or glass plate, with which we cover a suitable bowl containing a small amount of acetic acid. In a few minutes the epithelium can be removed in large pieces from the basement membrane.

Potassium Hydroxide enjoys quite a reputation, and is prepared by adding to 32.5 gms of caustic potash 67.5 cm^3 of water. The mixture is agitated well in a beaker until the potash is entirely dissolved. Since much heat is generated in this process the glass beaker must be kept in cold water. This strong solution will isolate non-striated and striated muscle fibres in fifteen to thirty minutes, but shrinks such tissues considerably.

Hydrochloric and Nitric Acids.—For the isolation of the tubules, ducts and alveoli of different glands, concentrated mineral acids are by far the best, such as nitric acid and hydrochloric acid, which are allowed to act on thin sections for from twelve to twenty-four hours.

Mechanical Maceration.—After tissues have thus been prepared by chemical agents, they can be isolated mechanically, e.g., after epithelia have been treated by the above-mentioned alcohol, they are thoroughly shaken in their container and the coarse pieces of tissue are removed. After a few hours the cells will settle on the bottom of the glass and can be taken out with the pipette. Sedimentation, however, is better and quicker accomplished by centrifuging.

When a mucous membrane has been prepared with acetic acid vapors, small pieces of the epithelial lining are picked up with a scalpel or spatula, and the cells thereafter isolated on the slide with teasing needles or by stirring.

Muscle treated with potassium hydroxide solution is transferred to pure glycerine and tincture of iodine is added until, on shaking, the fluid ceases to be decolorized. The next day the specimen can be transferred to water and the fibres separated by vigorous shaking.

When dealing with glands subjected to mineral acids, small pieces are taken and spread carefully on the slide in thin glycerine (1 part of glycerine to 2 parts of water).

THE CHOPPING METHOD

The Chopping Method.

Although somewhat primitive, this method yields excellent results and has the advantage of being less destructive to the tissues than other methods. It is especially indicated in the examination of the retina and striated muscle, no matter whether a fresh or a fixed specimen is used. It can, however, be employed for other tissues and organs.

A small piece of the organ is placed on the slide, and either in the dry state or after moistening the same it is carefully chopped with a sharp razor. Small particles will be found to adhere to the knife, and must be removed from time to time. The operator must take care not to spread the material all over the slide, but aim to keep it as closely together as possible. In this fashion

excellent transverse sections of the retina as well as of muscle fibres are obtained with the additional convenience of enabling us to study innumerable longitudinal sections.

THE CUTTING METHODS

Cutting of the Fresh Specimen.

As observed in the dissociation method of fresh specimens, it is also difficult to make cut sections, unless the material has previously been subjected to a hardening process. An object must possess a considerable degree of hardness to make a thin section possible, and in the natural state tissues are endowed with very little stability, excepting very few. Either they are much too soft and do not furnish enough resistance to the knife, as is generally the case, or the deposits of lime salts make them so hard that the knife is unable to penetrate (e.g., bone, tooth, cartilage of older individuals). A really suitable consistency in man and the mammalia is found only in the cartilage of the young.

Cutting of the Fixed and Hardened Specimen.

When dealing with a fixed specimen the prospects are much more favorable, most fixing agents acting to a certain degree in a hardening capacity. It is for that reason, that fixation and hardening are often used promiscuously. This is not entirely justified, since we have fixing solutions which not only fail to harden the specimen, but, under protracted use, even decrease their consistency, at the same time fulfilling all their requirements as fixatives. The best hardening is obtained from the prolonged use of alcohol, which is for this reason often continued after the fixation has been completed; e.g., alcohol formerly was extensively and to-day is to some extent used for preparation of the central nervous system.

Frozen Sections.

Cuts obtained in the said fashion, by hardening with alcohol, do not, however, fulfil all the requirements, and the necessity arises to find other more efficient means for the hardening of our objects. The oldest method, known for nearly a hundred years, is ***freezing.*** The specimen is frozen and sections are then made. This, the oldest hardening process, is used to this day with good results.

Embedding.

In this method our specimen is saturated with a substance, originally solid, which has been liquefied in a certain manner and will afterward again enter into the solid state. Of these substances paraffin and celloidin play the most important rôle to-day. To embed a specimen in paraffin, the latter must be heated to above its melting-point. The specimen immersed in this molten paraffin will become saturated and, after cooling, will attain a consistency which is mainly dependent upon the melting-point of the kind of paraffin selected. Celloidin, on the other hand, is liquefied by dissolving it in a mixture of alcohol and ether. Here, too, the specimen is immersed in the solution, and

hardening is secured by the evaporation of the solvent and suitable after-treatment.

Cutting Instruments.

Formerly the ***razor*** was used exclusively; it requires, however, great skill and an excellent knife to follow this practice. Hence the razor is not to be recommended to the beginner. Modern times have evolved instruments which make it possible for him to make useful sections without special skill. Such instruments are designated as ***microtomes.***

Following we will discuss in detail the three most important cutting methods.

Frozen Section Method

It is a well-known fact that freezing does not in any way injure the greater number of tissues, not even the highly developed organisms. We need only call attention to the experiments of *Raoul Pictet*, who froze frogs to a temperature of 28° C. below the freezing-point, so that they were as hard as glass, and yet after thawing them up, the animals recuperated and lived. Muscle of mammalia can be exposed to a temperature of —50° C. for a certain length of time; it will contract vigorously after the temperature has been brought up to normal again. Freezing fixed specimens does not deteriorate the latter in the least. It is evident therefore that the freezing process furnishes an excellent and unharmful method of hardening.

In former years it was a difficult or expensive task to secure the low temperatures necessary for the freezing, but all obstacles have been surmounted by the simple and inexpensive method found in the use of ***solid carbonic acid.***

Preparation of
Solid Carbonic Acid.

The solid acid may be prepared from the commercial liquid carbonic acid in the following manner: Use a bag made of strong silk velvet or chamois, which is open on one side, where it can be drawn together after the fashion of a tobacco-pouch. After taking off the cap, which closes the carbonic acid drum, this pouch is drawn over the mouth of the drum and secured tightly. The tank is placed on a chair, the head being slightly lowered, and the valve is opened for about ten to fifteen seconds. After removing the bag we will find it filled with a light white snow, the solid carbonic acid. The snow is collected on a cloth and thence transferred into a wooden container, provided with a funnel, and here it is ground together and compressed with hammer and rod (Fig. 19 *a*). After a few seconds a cartridge of carbonic acid can be pressed from the wooden container, which can be kept for hours in an isolated glass cylinder, the so-called *Dewar*—or thermos bottle (Fig. 19 *b*).

The Freezing Cylinder.

To freeze a specimen, the aim must be to guide the cold furnished by the cartridge exclusively to and into the specimen. To accomplish this purpose a ***freezing cylinder*** (Fig. 20) is used. It consists of a metal cylinder (*a*), which contains a *Dewar* bottle (*c*), the latter being protected by a felt

coat (*d*). The *Dewar* bottle is a double-walled glass cylinder, the walls of which are silvered, the space between the walls having been reduced to a vacuum. This serves to reduce the loss of heat by conduction and radiation to a minimum. The metal cylinder is closed on top by a threaded cover, on the under surface of which we find attached a second metal cylinder (*k*), which projects into the cavity of the *Dewar* bottle. In this smaller cylinder the carbonic acid cartridge is placed from below and the cover *l* is tightly adjusted. To the inner side of this cover a spiral spring is attached (*m*) which serves to press the carbonic acid tightly against the undersurface of the upper large cover. Into the latter the freezing-table proper is screwed (*o*), being separated from the outer metal cylinder by a hard rubber ring.

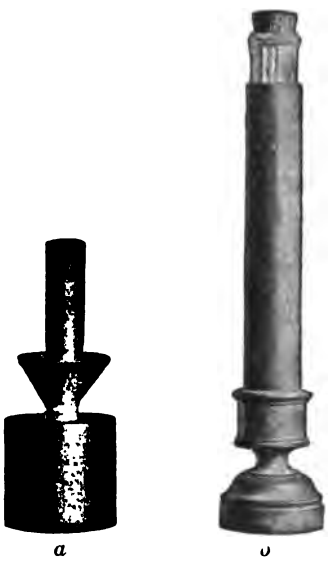


Fig. 19.

Press, *a*, and Retainer, *b*, for Solid Carbonic Acid

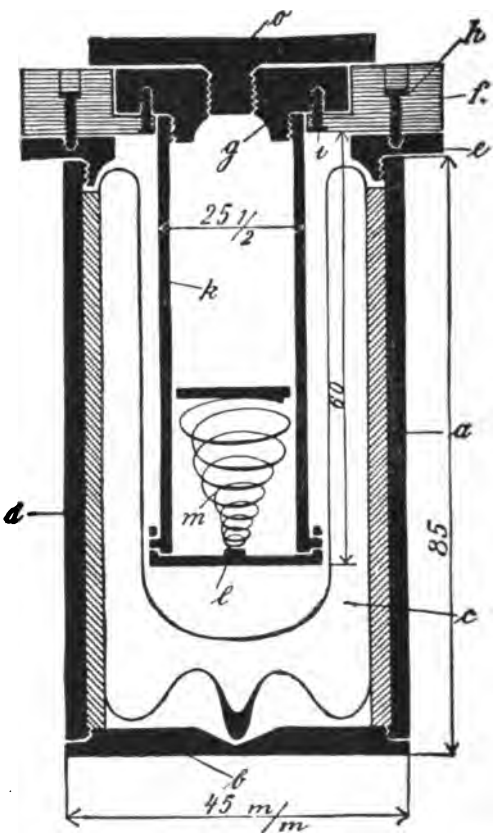


Fig. 20.

Construction of Freezing Cylinder. Explanation in text.

The Freezing Microtome.

The described apparatus can be adjusted to most **microtomes.** We prefer an instrument furnished by *M. Schanze* (Leipzig), which combines stability with greatest precision and is not very expensive. In Fig. 21 we see the instrument with the freezer attached. It rests on a heavy cast-iron plate (*gpl*), from which arises a vertical plate (*vpl*), which in turn gives off a third plate at an angle of 45°. This latter, together with the upper part of the vertical

plate, thus forms an horizontal track in which glides the knife-carriage, a heavy triangular metal block (*mschl*). Its upper surface serves for the attachment with bolt and nut of the knife (*m*), which is provided with a forklike handle. On the other side of the vertical plate (the one turned toward us) we find the specimen-carrier. It glides on two vertical rails, the left only being visible in our picture (*opl*). A lever (*h*) serves to move the specimen-carriage (*oschl*) up and down. In close approximation with the specimen-carriage is the specimen-holder. It consists of two shafts, placed at right angles to each other, which can both be secured by means of a screw (*fschr*). The specimen-holder proper (*oh*) is connected with the upper shaft; it mainly consists of a ring, which receives the freezing apparatus (*gfr*). The latter can be raised or lowered in the ring by means of a screw. The finer adjustment of the specimen-carriage can be procured by the use of the micrometer-screw. It is only partly visible in our picture, the lower end being hidden in

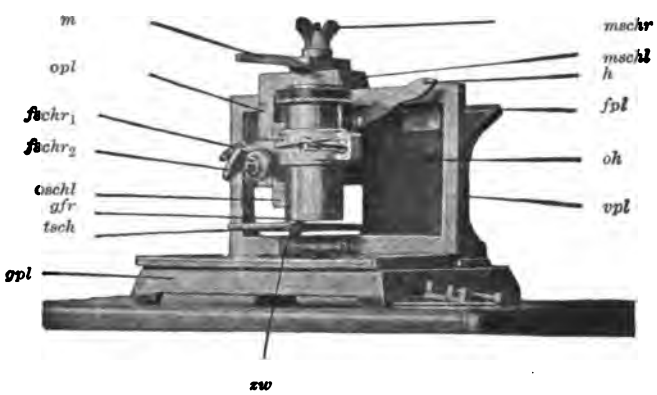


FIG. 21.

The Freezing Microtome. Paraffin Clamp at the Right of the Microtome.

the clamp (*zw*), while the specimen-carriage rests on its upper extremity. The section scale (*tsch*) serves for the adjustment of the thickness desired. It is divided into 50 whole or 100 half degrees, respectively. If the disc is turned one whole degree toward the left, the specimen will be raised 10 μ, so that thicknesses of 5 μ and its multiples can easily be obtained.

The Practical Use of the Freezing Microtome.

Unscrew the cover of the freezing apparatus, which, being intimately connected with the freezing cylinder, will lift the latter out, then open the lock-cover on the lower end of this cylinder, insert a cartridge and close it again. The cover is now screwed on again and the specimen, moistened with a few drops of water or normal saline, is placed on the freezing-table. After five to ten minutes it will be entirely frozen. The knife should be attached to the knife-carriage so that it stands vertical to the long axis of the microtome. The screw designed to hold the knife (*mschr*) must necessarily be drawn tight. By rotating the freezing cylinder up and down, the upper surface of the specimen is closely approximated to the edge of the knife, the apparatus fixed in this

position, and we can now proceed to cut. The knife-carriage is moved forward with the right hand, evenly and without exerting any pressure. Preceding each cut the left hand turns the section scale a corresponding number of degrees to the left, according to what thickness may be required. The sections obtained either cling to the knife, becoming soft, or they drop to the freezing-table, where they will lie in the frozen state. All these manipulations are much easier executed than they are described.

How to Obtain a Favorable Consistency.

The consistency of our specimen is naturally of prime importance for the securing of useful sections, and the former is mainly dependent upon temperature, though also influenced by the structure of the specimen. Our apparatus produces a temperature of —12 to —15° on the freezing plate. It is generally used for fresh specimens, those retaining their own natural tissue juices; for objects that have been taken out of water and are therefore also saturated with it, the temperature is too low, since such objects would split on cutting. This objection can be obviated by placing the specimen in a fluid having a lower freezing-point than water, e.g., very dilute alcohol or, better yet, dilute formalin, where it should be kept for several hours. The author recommends 5% formalin, which should not be removed before placing the object on the freezing-table. This procedure will almost invariably give good results, and only very seldom will one have to resort to 10% formalin.

Time and care should be taken in cutting, as our apparatus will keep the specimen frozen for at least one-half hour, and if signs of thawing should be noticed, it will only be necessary to insert a fresh carbonic acid cartridge, and the work can be continued in leisure.

Thickness of Sections.

As regards the thickness of sections, we can easily obtain cuts of 5—10 μ, even with a large cutting surface, by the use of this freezing microtome; but we do not recommend such thin sections, unless especially required, since the further treatment of sections becomes more difficult the thinner the cut. Sections of 15—25 μ thickness should answer our purpose and will always show even the finest structural details, if they are stained in the proper manner.

Treatment of the Sections.

If the section curls up and drops to the freezing-table, the transport is very simple; it is grasped with a pair of fine tissue forceps and thrown into a bowl of water, where it will generally unfold itself without any aid. Should the section adhere to the knife and thaw, it will have to be brushed off with the tip of the finger, which is then dipped into the water. Each section may be taken off separately, or the operator may wait until a number have collected and then take them off *en masse*. The finger should be thoroughly dried each time to avoid bringing water to the cutting surface.

When handling a very small and thin specimen it is best to first freeze enough water to make a layer of ice 2—3 mm in thickness, on top of which the object is placed. If we want to remove the latter again, the freezing-table must be unscrewed from the cover and water poured on its reverse surface for a few

seconds, or the whole of it dipped in water, after which the specimen, still frozen, can be detached.

The Paraffin-Section Method

Much more complicated than the freezing is the ***paraffin-section method.*** The first consideration will be to embed the specimen in paraffin, to wit, not only to give the object a coating of paraffin, but to saturate every part of the specimen. This is practicable only if the specimen is first thoroughly penetrated by a liquid, which is a good solvent of paraffin, and for this reason quite a complicated preparatory treatment is necessitated.

Paraffin.

As paraffin are classed mixtures of hydrocarbons of the general formula of $C_n H_{2n+2}$ in which the value of n fluctuates between 20 and 27. It is a coal-tar product, but has also other sources, e.g., the hardest variety has its origin in ozocerite.[1] It is a white, fatty substance of a specific gravity of 0.9. The melting-point may be anywhere between 40° and 85° C., according to the origin of the paraffin. Paraffin is absolutely insoluble in water and almost insoluble in cold alcohol. It is soluble in carbon disulphide, chloroform, benzine, benzol, xylol, toluol, petroleum ether, etc. The boiling-point is 300°.

Preparatory Media.

The reader will notice that the above-mentioned solvents of paraffin do not mix with water in any proportion, but can easily enter into a mixture with alcohol in any proportion; if, therefore, we want to saturate our watery specimen with such a solvent of paraffin, which we will call a ***preparatory medium,*** it will first be necessary to dehydrate the tissues by the use of alcohol. Let us select ***chloroform*** as a standard preparatory medium, since it possesses many advantages over the others. The preparatory treatment will be as follows:

Dehydration.

The fixed and washed specimen, which we suppose to be in water, is gradually transferred to alcohol of increasing strength. Starting with 10% alcohol we increase at the rate of 10%. We make the desired strength by diluting absolute alcohol with the corresponding amount of water. The length of time required for each grade of alcohol depends wholly on the size of our specimen. Pieces of 1 cm^3 will require approximately twelve hours, while two to three hours suffice for smaller pieces. The specimen should always stay in its container, for which a wide-mouthed bottle will serve best; the latter should be corked and labelled as to origin of specimen, manner of fixation, and the respective alcohol in use. In this fashion specimens, lest they be very large, can be dehydrated in five days, if at the end we rechange the absolute alcohol once.

The introduction of the specimen into the preparatory medium should also take place gradually. As a rule, three steps will suffice. First, 3 parts of

[1] ὄζω= smell,— κηρός= wax; a natural paraffin, found in Galizia, having a green, brown or red color and an odor of petroleum.—(The Translator.)

alcohol are mixed with 1 part of chloroform, then equal parts are used and, finally, 3 parts of chloroform to 1 part of alcohol. In each of these mixtures the specimen stays from six to twelve hours, although a longer period will do no harm, after which it is transferred to pure chloroform overnight.

The saturation with paraffin can only be effected by placing the specimen in paraffin, previously heated until liquid, and by keeping the same liquid during the entire process. To do this we must use a ***thermostat,*** similar in construction to those used for culturing bacteria and for the artificial breeding of chicken eggs. Fig. 22 represents such an apparatus, which is suitable for our purpose. It is made of strong copperplate, has double walls and a door, the space between the walls being filled with water, or, better still, with acid-free glycerine. A water-gauge will indicate the depth of the water. A second box surmounts the former, also equipped with a door, but having only simple metal walls. The temperature obtained in either compartment is indicated by separate thermometers. The heating is done by a burner placed beneath the lower box; this burner has a safety device which shuts off the gas supply in case the flame should suddenly be extinguished. Before entering the burner the gas passes through a regulator situated within the double wall. The temperature can thus be regulated at will, so that only so much gas will be admitted to the burner as is necessary to obtain the desired temperature. There are also similar thermostats on the market which can be heated with petroleum or by means of electricity. The temperature of the thermostat should exceed the melting-point of the paraffin in use by about 1—2°. As will be shown later, we select, as a rule, a paraffin having a melting-point of 54—56°, and should therefore regulate the temperature of our thermostat to 56—58°. This temperature will be maintained in the lower compartment, while in the upper, which is indirectly heated from the lower, the temperature obtained will range between 40° and 45°.

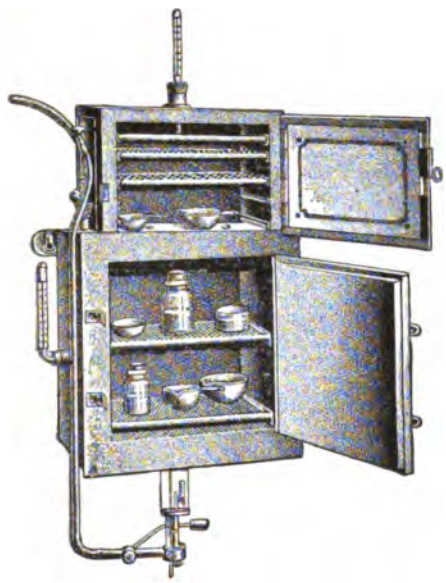


Fig. 22.
Thermostat for Embedding in Paraffin.

The Melting-Point of Paraffin.

An important factor in the paraffin method is the correct selection of the paraffin as regards its melting-point. The following considerations should be borne in mind: The thinner we desire the sections to be made, the harder the paraffin must be, or, in other words, the greater must be the difference between the room temperature and the melting-point of the paraffin; e.g., with a paraffin

of 52° melting-point sections of 5 μ or even thinner can be made in a cool room, which would be impossible in a warm room or on a hot summer day. Again, in the former case it would be hard to make sections as thick as 25—50 μ, which would be an easy task in the latter instance. We might say that the preparation of thin sections requires a difference between room temperature and paraffin melting-point of approximately 40°. If this difference decreases, thin sections will be compressed during the cutting process, and will consequently be deformed. For thick cuts the difference must not exceed 30—35°. If it is raised in this instance the section will break.

The best plan is to select two sorts of paraffin, one, the soft paraffin, with a melting-point of 42°, and the other, the hard paraffin, with a melting-point of 56°. The latter is used for thin sections, while in the preparation of thicker sections we will have to add more or less of the former quality to the latter. As a receptacle for the paraffin, round dishes with a flat bottom, lined with porcelain or enamelled tin, will serve best.

Saturating the Specimen with Paraffin.

Great care must be used and the temperature changes must be gradual when the specimen is to be transferred from the preparatory medium into the liquid paraffin. After the specimen has been saturated with chloroform, the excess of the latter is decanted until only sufficient is left to cover the object; then a few pieces of soft paraffin are added and the uncovered glass is placed in the upper compartment of the thermostat. After a few minutes (15 to 30) the paraffin will dissolve and a few more pieces can be added, and so on until a sufficient quantity of paraffin is in the glass to cover the specimen, while in the meantime the chloroform is slowly evaporating. The latter process will be complete the next morning, and, instead of chloroform, our specimen will now be saturated with the liquid soft paraffin.

During this time we can prepare our liquid hard paraffin in the following manner: A suitable basin is filled to the brim with small pieces of hard paraffin and placed in a lower compartment of the thermostat. During the night the paraffin will be liquefied. The specimen is now brought from the upper division into the lower and after reaching the temperature of 58° in about half an hour, it is transferred to the hard paraffin with a heated spatula. It should not be kept there unnecessarily long, but not less than two hours, when reasonably large. Sudden temperature changes are deleterious, more so than too prolonged, but gradually completed exposure to hard paraffin.

Paraffin Embedding.

This brings us finally to the ***embedding*** process proper. This can be accomplished in little paper boxes, watch glasses, or, better than either, in small metal boxes. They consist of two brass strips bent at right angles, one limb being shorter than the other (Fig. 23). When the two strips are brought together they form, with the underlying glass plate, a little box, which is open on top and the length of which can be adjusted as desired. The glass plate and inner surfaces of the brass are thinly

FIG. 23.
Frame for Embedding in Paraffin.

coated with vaseline. The box is now filled with paraffin and the specimen immersed in the same, and then deposited in a position most suitable for cutting. Several specimens can be embedded in this manner in one box by placing them at a proper distance.

Cooling of the Block.

The paraffin must needs be cooled now, and this can easily be accomplished by placing the box including the glass plate in a large basin containing cold water. Care must be taken here, since the lighter paraffin tends to rise to the water-surface. This can be overcome by carefully inclining the bed, and let-

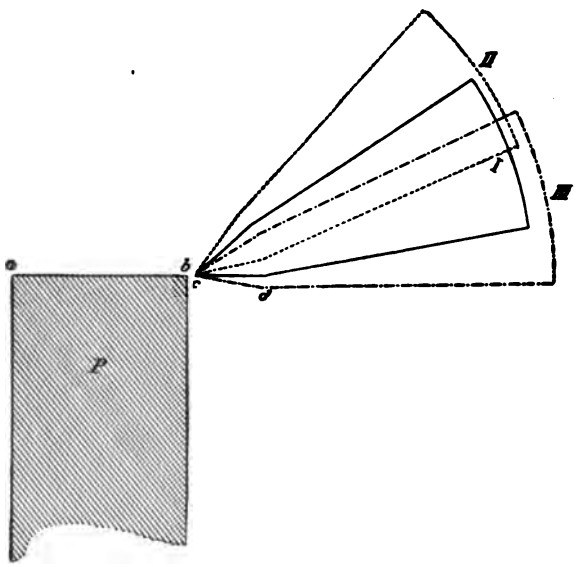


FIG. 24.
Position of Knife in the Cutting of Paraffin Sections.
I, correct; II, too acute; III, too obtuse.

ting the water flow over it gradually. After a few hours our block is completely solidified and can easily be removed from the bed.

The Microtome for Paraffin Sections.

The microtome, mentioned previously, can also be used here; we simply remove the freezing-table and substitute the paraffin clamp, which is depicted in Fig 21, lying next to the microtome. The paraffin block itself may be properly shaped and adjusted in the clamp, or it can preferably be mounted first on a small wooden block and the latter fastened in the clamp. Of course the clamp can be left out all together, and the paraffin block molten directly to the freezing-table.

The Cutting of Paraffin Sections.

This proves more difficult to the beginner than the preparation of frozen sections, for the reason that in this method the ***position of the knife*** is of

great importance. While it does not matter whether our knife is in an oblique or sloping position, the angle formed by the cutting edge and the horizontal plane is the vital issue. The microtome knife is wedge-shaped and acts like a wedge, the surfaces becoming abruptly converging near the cutting edge, thus practically forming a composition of two wedges, the two converging surfaces near the edge receiving the name of cutting facettes. If we picture an object being placed before this double wedge (Fig. 24), it will be possible to make a section only if the lower facette *c d* either coincides with the cutting surface *a b* (I) or forms with it an angle above 180° (II). If the angle should be less than 180° (III) no cut will result, since the facette will simply glide over the surface of the object, polishing it smooth. Only, if the specimen is elevated enough to let its surface be on a higher level than the knife-edge, an irregular section may be accomplished. To make a correct cut, the knife must be rotated upon its long axis and adjusted more steeply. Larger instruments possess for this purpose a separate knife-holder, but we can accomplish the same purpose by using two small wooden plates, placing one under the anterior, the other over the posterior handle of the knife. A metal ring is placed on top and the whole fastened tightly in the clamp. Several sizes of wooden plates should be kept on hand, so that an appropriate thickness may be selected, since the angle of the cutting facettes changes with almost every honing of the knife. Again, the position may be too steep; this will be indicated by the presence of numerous breaks in the section, running parallel with the edge of the knife.

The Curling of Sections.

All paraffin sections have more or less tendency to curl upon themselves. This must be obviated by the use of a camel's-hair brush. While the right hand moves the knife-carriage, the left holds the brush, and as soon as the knife enters the substance of the block, but not before, the section is gently pressed against the knife.

Mounting of Sections.

The next step is the mounting of the section on the slide, although the specimen may also be mounted on the cover-glass or on isinglass. The cover-glass or slide or isinglass, as the case may be, must first be thoroughly cleansed. If new, they should first be moistened with dilute (20—30%) alcohol, and dried with a clean cloth. Then a minute drop of egg albumin glycerine (cut up the white of an egg, filter and mix with an equal part of glycerine and a large crystal of thymol) is placed on the centre of the slide and spread thoroughly with the tip of one finger. A large drop of water is quickly placed on this layer of egg albumin and allowed to spread and the section placed therein. The slide is now held over a flame, the heat being estimated by the hand, until the sections flatten out completely, without being burned, the superfluous water is then decanted, the sections arranged, and the slide placed in the upper part of the thermostat to dry. A few hours will suffice, after which the sections will be found to adhere completely.

For sections which are not to be stained another method is more suited. The slide is coated with a thin layer of a mixture of oil of cloves and collodium (collodium 1 part, oil of cloves 3—4 parts), the sections are pressed down

with a brush or dry finger-tip and can then be transferred to xylol to dissolve the paraffin.

The Celloidin Section Method

Celloidin.

Celloidin, a highly concentrated solution of guncotton, dinitrocellulose, in a mixture of alcohol and ether, is sold in the form of rectangular plates of a finger's thickness, having a consistency of cartilage. They are saturated with alcohol and packed in hermetically sealed tin boxes to avoid drying.

The Preparation of the Celloidin Solution.

To prepare a solution suitable for embedding, the celloidin must first be carefully dried. The plate is cut into strips, which are in turn divided into smaller pieces and put in a glass beaker. After protecting the contents from dust by covering with a sheet of paper, the beaker is put in the thermostat. After two to three days the celloidin particles will have dried and shrunk to opaque hornlike chips. Care must be taken in the following steps, as the celloidin in this dried state is explosive. The chips are put in a glass-stoppered bottle (of 800—1,000 cm^3 volume), which must be absolutely dry, and are covered with 250 cm^3 of absolute alcohol. The mixture is frequently shaken or stirred with a glass rod. After twenty-four hours the chips will be softened and swelled to a glassy mass. 250 cm^3 of water-free ether are added and, if promptly stirred and shaken, the solution will be complete in from two to three days. We now have a mass of honeylike consistency, the ***celloidin stock solution.*** From the latter we prepare two dilutions: ***Celloidin I*** (1 part of stock to 3 parts of alcohol-ether), and ***Celloidin II*** (1 part of stock to 1 part of alcohol-ether). Both solutions should be kept in well-corked bottles.

Dehydration and Saturation.

As regards celloidin embedding we find that here, too, thorough dehydration is of prime importance; we therefore have to gradually transfer our specimen into stronger and finally absolute alcohol, the latter being changed at least once. It is then brought into a mixture of equal parts of absolute alcohol and water-free ether, and from there into Celloidin I, i.e., the thin celloidin solution. The length of time spent in the latter (of course in a well-closed bottle) depends entirely on the size of the specimen. For very small objects two days are sufficient, larger ones must remain a correspondingly longer period up to two or three weeks. From this thin solution the specimen is transferred to the thicker Celloidin II, and thence to the stock solution, remaining in each an equal length of time.

Preparation and Preservation of the Celloidin Block.

After saturation with the stock solution the specimen with the retained celloidin is placed on a correspondingly large dry wooden block, exposed to the air for about ten minutes and preserved in a large quantity of 70—80% alcohol. After being acted on by the alcohol for twenty-four hours the specimen will have attained a consistency adapted for cutting. Before using, the wooden blocks must be treated for several days with 95% alcohol, preferably

warm, to free them from tannic acid and resin. ***Stabilit,*** a material often used in electro-technique, may be used instead of wood; it can easily be divided with a saw.

Preparation of the Celloidin Section.

The microtome, previously mentioned, with attached clamp, can readily be used for the preparation of celloidin sections. The knife is adjusted as obliquely as possible, forming an acute angle with the long axis of the microtome, so that in cutting the entire edge is made use of. This presupposes, of course, that the cutting edge is immaculate. During the cutting the knife is constantly moistened with 70—80% alcohol by means of a large camel's-hair brush, so that the sections float on the edge. Tissue forceps will serve to transfer the sections from the knife to the alcohol dish. As soiling of the microtome with alcohol cannot be avoided during all these procedures, a thorough cleaning of the instrument afterward is essential.

SELECTION OF THE CUTTING METHOD IN SPECIAL CASES

Each of the methods described has its own merits as well as disadvantages. The freezing method excels the other two without doubt, on account of its ready execution and rapidity. Here our specimen does not come in contact with any extraneous reagents, excepting the formalin solution, and therefore this represents the most conservative method, altering the staining properties to the least extent. On the other hand, this method presents a great disadvantage, met in objects in which the component parts are only loosely connected or are separated by interspaces, where this connection is easily, often unavoidably, destroyed, and this fault will not be remedied as long as we fail to discover a suitable embedding method for fresh objects or those kept in water.

The paraffin method is doubtless the most aggressive of the three methods, necessitating not only a thorough treatment with alcohol, but also the heating of the specimen to a considerable degree. The advantage in this procedure lies in the fact that specimens so treated allow of the most delicate cuts, and that in these sections the original position of the parts is absolutely preserved, and furthermore that the after-treatment is the easiest imaginable. Its present supremacy in our histologic and zoologic laboratories is due to these advantages.

Midway between freezing and paraffin methods stands the celloidin section method, which avoids heating the specimen, but requires a longer saturation and the most careful manipulation. Thin sections, i.e., 5—10 μ, may be obtained in either of the three methods. The thinnest are undoubtedly furnished by the paraffin method, at least when the specimen is small. When dealing with larger objects the freezing method will answer as well or often better.

The reader can deduct from these suggestions which method be best suited for a particular case. We always give the freezing method the first rank, as being the simplest and least aggressive, and choose the others only when sec-

tions of the finest precision are wanted, or when the structure of the tissue calls for embedding.

APPENDIX TO THE PREPARATION OF SECTIONS

DECALCIFICATION

The hard structures of the human body, viz., bones and teeth, due to deposits of lime salts, possess such firmness that they resist cutting. To render them soft, we must aim to remove these salts by treating them with their solvents, viz., acids. This process has received the name of ***decalcification.***

In many cases the fixing solution can be used as such by protracting its application; e.g., chrome-osmium-acetic acid and sublimate-nitric acid are excellent decalcifiers and yield good results if small specimens are suspended in large amounts of the liquid and the latter is changed frequently.

As a general rule, however, we recommend to fix the object first thoroughly and then expose it to the action of a decalcifier. As such only those acids can be considered which can change the carbonates or phosphates of the hard structure in question into their respective soluble salt.

Among these acids nitric again ranks foremost. As a decalcifier we use it in a 5% watery solution, immersing the fixed and washed specimens in large quantities of same. To expedite decalcification, the specimen is frequently shaken, or it may be suspended in the upper layers of the fluid and the latter changed repeatedly. The duration of this process of course depends on the size of the object and the amount of lime contained in it. In order to test the progress of decalcification the specimen may be pierced with a needle or an attempt at cutting with the razor may be made, unless such procedures are contraindicated for other reasons. With fair sized pieces of only moderately old bone two to four days will usually complete the process, for whole teeth eight to ten days will suffice. After decalcification the acid must not be directly washed out with water, but the specimen first immersed in 10% formalin (which is changed frequently) or in 5% *Glauber* salt solution (also to be changed repeatedly) for twenty-four hours, after which the washing can be done without any harm resulting.

Of the other decalcifiers we will only mention the trichloracetic acid, the method of procedure being identical with that of nitric acid.

STAINING METHODS

Staining.

By ***staining*** we mean that process by which an unstained body, treated with the solution of a stained body, appears stained itself. The color resulting will usually be the same as that of the solution used; however, under certain circumstances this may not be the case. Dyeing is a primeval art, owing its development to the beauty sense of mankind. The methods employed for the staining of our microscopic specimens are largely drawn from the practical dyeing process, but not with a view as to beauty in colors, but solely because

the staining is an important, if not the most important, aid in the diagnosis of tissues and their component parts.

Stains.

All stains used in microtechnique are organic compounds, viz., they contain carbon and hydrogen. They either owe their origin directly to the animal or vegetable kingdom, or, as in the majority of cases, they are artificially prepared (synthetic), so that we must differentiate between ***natural and artificial*** stains.

The Essentiality of Staining.

It has not been decided as yet with accuracy what the process is that causes an organic body to appear colored, i.e., to let certain rays of the spectrum pass through, and reflect others, and absorb still others, but so much we may safely assume, that this property is founded in the chemical constitution of the body, and a number of atom groups are already known which possess the capability, when entering an unstained body, to change the same into a dye. In the same manner the ***essentials of staining*** have remained rather obscure. Three theories have so far been expounded. The ***mechanical theory*** assumes that physical powers alone are concerned in driving the staining solution into the tissues, namely, osmosis and capillary attraction. Once in the tissues the dye is condensed by absorption in a similar manner, as animal charcoal may detract all the dye out of a stain solution and render the latter colorless. The ***chemical theory,*** on the contrary, claims that chemical, saltlike compounds are formed during the process of staining between the stain, respectively, its components and the albuminous substances of our specimen. As we have discussed previously, these albuminous bodies partly take on the character of acids, partly that of bases, and it therefore seems quite reasonable that by their combination with the stain acids, respectively, stain bases of our dyes, saltlike compounds result. The third theory finally considers the stained preparation as a solidified solution of the stain in the tissues. This is not the place to enter into a discussion of the merits of each of these theories. Suffice it to say that without doubt chemical processes are concerned in the phenomenon of staining, which, however, do not exclude the possibility of processes of a physical nature occurring at the same time.

Preparation of the Color Bath.

A stain to be of use in our work must be soluble. ***Solvents*** are primarily water, but also alcohol, rarely glycerine; furthermore, salt solutions, weak acids and alkalies are all used. To increase the staining power of some of the watery solutions of dye, aniline is added, or instead of dissolving the coloring matter in water, we use aniline water, which can be prepared by mixing a small amount of aniline with distilled water, thoroughly shaking it and filtering the opaque mixture through a previously moistened filter.

Concentration of the Stain.

As a rule, we prepare staining solutions of a fixed strength, i.e., a certain amount of dye is dissolved in a given amount of solvent, so that all of the dye

will go into solution. The dissolving takes place either at room temperature or the solvent is previously heated. Often, however, we use the so-called concentrated solution, which is made by introducing an excess of the dye into the solvent and shaking repeatedly; after a while we find above the sediment of the excess a solution of maximum concentration. Owing to the fact that in most cases the dyes are more highly soluble at a higher temperature than at some lower, the strength of such solutions must needs fluctuate with the temperature in which they are kept. When using concentrated solutions the decanting must be done with great care, to avoid picking up some of the small solid particles of dye from below.

It is difficult to say in general which concentration would be apt to yield the best results. We often hear it mentioned that thin solutions work better than the concentrated. That may be the case with some certain dyes, but cannot be adopted as a general principle. In short, the concentration must depend upon the nature of the dye and the particular case in which it is to be used.

Temperature of the Staining Bath.

As a rule, we stain at room temperature, but special circumstances may demand a higher temperature, when the staining solution must be warmed; this is identical with the custom of heating the solutions in practical dyeing. Beyond doubt the heated dye will penetrate tissues in a shorter period of time and the staining will be more intensive than if lower temperatures prevail.

Staining of the Living Specimen.

Living specimens, as well as preserved, may be stained. The former is called ***vital staining*** or ***staining in the living state.*** A vital staining, in the strict sense of the word, can only be executed in the living animal body itself, by incorporating into the latter the desired dye in some appropriate fashion. Vital staining is of great importance in research, and, although still in its infancy of elaboration, the results obtained have been brilliant. In the higher animal the incorporation of dye may be accomplished by means of ***feeding,*** the dye being mixed with the food. The dye having been dissolved in the chyme, absorption takes place in the intestines. A quicker method is to inject the watery solution ***hypodermatically,*** and finally the ***intravenous method*** may be resorted to, by introducing the stain into a vein of the anæsthetized animal. Of course the dye must not be poisonous enough to cause a cessation of heart action. If such should be the case, the animal is allowed to bleed to death, after which the circulatory system is flooded under pressure, the place of introduction being the heart or an artery. The latter method can be used to good advantage in the human body, e.g., an amputated extremity.

Staining of the Surviving Specimen.

We often speak of vital staining, when small sections are taken from the living or ***recently killed animal*** and subjected to the action of a staining fluid. It is a question whether we have here really vital staining, since there are no criteria which would show us that such a section of tissue or its com-

ponents at the moment of staining are still living or have died, a question which might also enter into the vital staining proper. Hence it might be wiser to designate such procedures as staining of fresh specimens, i.e., such as are not preserved. Weak solutions should be used for this purpose and, if possible, the dye should be dissolved in some indifferent fluid, e.g., normal saline or *Ringer's* fluid. We can either take small particles of the organ with scissors or razor, or we can first make frozen sections of these particles and stain the latter. The chopping method is also useful, the specimens being quickly chopped on the slide, after which the stain is added, the specimen put in a moist chamber and the whole eventually placed in the thermostat at a temperature of 38—40°.

Staining of the Preserved Specimen.

The staining of ***preserved*** objects is far more common. Here we must always bear in mind that our fixation and preserving process causes invariably radical changes in the chemical composition of the albumins, which make up cells and tissues. One should therefore be guarded in promising results, which are to be obtained with these methods.

Lump and Section Staining.

Two separate methods of procedure are to be distinguished in the staining of preserved objects; on the one hand we may stain larger or smaller ***lumps*** of ***tissue*** in toto, on the other we subject the sections made of the piece of tissue to the dye. The lump staining requires a dye of great power of diffusion, unless we deal with membranous structures. Most of the watery solutions lack this quality, whence it is necessary, with few exceptions, to resort to alcoholic solutions when staining in lump is desirable. Another disadvantage of lump staining lies in the fact that we are not able to control the staining process sufficiently. For these reasons this method is used only seldom in histology, while embryology employs it frequently to this date. In section staining there is no difficulty in controlling the progress of the staining process and to interrupt it at the proper time, and therefore it has been adopted as the superior of the two methods.

Staining of Frozen Sections.

For the staining of ***frozen sections*** watch-glasses of different sizes with ground edges, convex on the surface and straight on the bottom, on which they rest, are most serviceable. As a cover a glass plate or a similar watch-glass of the same size may be used. The sections are placed into the staining fluid by means of a curved, well-polished needle or, better still, with slightly curved glass needles, which can be easily made from a glass rod or a thick-walled glass tube (barometer tube) in any desired size. It is important that these needles be smoothly polished; if they lack in even smoothness or present roughness in the least degree, sections may easily adhere and tear. The specimens are removed in the same manner. When the stain is opaque, difficulty in removing the sections may be encountered. When staining but a few sections, only a few drops of the solution are necessary; when staining a larger amount

in a correspondingly larger quantity of dye, the curved needle is guided along the bottom of the dish and the sections are fished out gradually.

Staining of Paraffin Sections on the Slide.

The staining of ***paraffin sections*** previously pasted on the slide is best performed in cylindrical glasses, which must be slightly higher and a trifle broader than our slides. The first aim will be to remove the paraffin from the section, since its presence renders staining difficult, although not impossible. A good solvent is found in ***xylol,*** $C_6 H_4 (CH_3)_2$, a waterlike transparent fluid, lighter than water and unable to mix with water in any proportion. The paraffin will dissolve in a few seconds, if the slide is lightly agitated to and fro, and if the sections are not too thick. In order to be able to transfer the specimen into our watery staining solution, we must now remove all the xylol from it, which is accomplished by the use of ***absolute alcohol;*** from the latter the sections are first transferred to a more dilute grade of alcohol, say 90%, before the staining proper can begin. First the watery stain will not penetrate the section, but after moving the latter to and fro a few times, it will enter the tissue. Practically it is best to have several of these glasses side by side, the first containing xylol, the second absolute alcohol, the third 90% alcohol, and the fourth the staining fluid. It is of advantage to insert a fifth glass between the third and fourth, containing water. Specimens are transferred from one fluid to the other, several slides being placed in one glass if necessary, by placing them in pairs with their backs approximated. The film sides of course must not be touched in any way in order to avoid laceration of the section.

Staining of Celloidin Sections.

Celloidin Sections are treated in a similar manner as are frozen sections, with this exception, that less care need be taken in transferring. The specimen is simply put from alcohol to water and thence into the stain. If the latter is an alcoholic solution, the water, of course, is omitted.

Simple and Multiple Staining.

Depending upon whether we stain with ***one*** or with ***several dyes*** we differentiate between ***simple and multiple staining.*** In multiple staining we naturally select dyestuffs which differ widely, in general, contrast-stains. When several stains are used ***in succession*** we speak of ***successive multiple staining;*** again, when a mixture of several dyes is used for staining we call the process ***simultaneous multiple staining*** or ***compound staining.***

Progressive and Regressive Staining.

If the staining process is interrupted at its height, i.e., the ***optimum*** of the process, we speak of ***progressive staining;*** in that case we need only wash out the excess staining solution from the specimen. When using the ***regressive staining*** method, we overstain the section intentionally, bringing it to the ***maximum*** of the process, the optimum being gained by treating the

specimen with a solvent of the dye used, or by partly destroying the stain. Such agents are known as ***differentiation agents.***

Substantive and Adjective Staining.

According to practical dyeing we can also differentiate between ***substantive*** and ***adjective*** method. The process is substantive when a simple solution of a dye is used ***without any adjunct agent.*** In the adjective method we have besides dye and specimen a third factor, without which the dye will fail to stain the tissue. Such agents are designated as ***mordants*** or ***bases,*** and we speak of a ***mordant dye,*** when it alone has little or no staining power, the latter only being developed when the base is added. Here, too, chemistry has given us the key to the relation existing between the composition of a dye and its staining power, inasmuch as we know that all dyestuffs containing two hydroxyl groups in ortho position are mordant dyes.

Mordants.

Agents of all sorts of chemical structures are used as bases in commercial dyeing, e.g., acids, oils and the product of the action of the former on the latter, tannins, metal oxides and others. In microtechnique we use almost exclusively metal oxides for this purpose. The mordant[1] dyes possess the property of forming with ***metal oxides*** saltlike compounds of high staining power, being soluble in water only to a small degree. Such compounds are designated as ***color lakes.*** We can prepare the latter in vitro, put them into solution in the proper manner, and then stain with this solution of the lake; i.e., ***simultaneous mordant dyeing,*** or we first let a solution of our base act on the specimen and stain it thereafter, ***succedaneous mordant dyeing.*** Then the lake is created in the specimen. Mordant staining plays an important rôle in microtechnique, the simultaneous process as well as the succedaneous.

Diffuse and Elective Staining.

The result of a staining process may be ***diffuse*** or ***elective.*** It will be diffuse if all the component parts of the specimen appear ***equally stained.*** Staining is called elective when either ***certain*** parts of a tissue are affected, while others remain unstained, or when all the parts are stained but differ in shade of the same color or under the most favorable circumstances appear actually in different colors. It is evident at once that diffuse staining is of small value, and that our aim must be to accomplish elective staining. This end may be accomplished in various manners, since experience has taught us that the different dyes have a different affinity to certain kinds of tissue, so that, when treated progressively, they are stained more quickly, while under the regressive method they retain the particular dye correspondingly longer.

A specimen treated with the simultaneous or succedaneous method will show that, under proper technique, certain dyes are taken up by certain parts of tissues; e.g., certain dyes only stain the nuclear chromatin, the basic sub-

[1] The terms, "mordant" and "base," are used interchangeably to represent the German *"Beize,"* the word mordant being of French origin: *mordre* = to etch.—(The Translator.)

stance of cartilage, mucus, certain kinds of cellular granulations; others, again, will affect the nucleoli, the protoplasm of the cell body, the hæmoglobin of the red blood corpuscles, the connective tissue shreds, the basic substance of bone, etc. We have thus found that the group mentioned first is stained by ***basic dyes,*** i.e., combinations of color bases with any desired acid, while the latter is affected by ***acid dyes,*** i.e., color acids or their alkaline salts; we may therefore call the first group of tissues or parts thereof ***basophilic,*** the latter ***acidophilic.***

Metachromatic Staining.

It is not always necessary to treat a specimen with two different colors, say red and blue, in order to obtain red in certain tissue elements and blue in others; we have certain chemically uniform dyes, which possess the property of staining in two different colors, or even will bring out different shades of these colors in various tissue components. Such dyes are known as ***metachromatic.*** Metachromasia is a virtue possessed by many, but principally by basic dyes, where the free base has a different color than the salt represented by the dye; e.g., not infrequently we find that a color base is staining red, while its hydrochloric acid salt will give a blue color. The latter we use as a dye, since the color bases are, as a rule, insoluble in water. If we now proceed to stain in the watery solution of such a blue dye, certain elements will be stained orthochromatic, i.e., in the color of the solution, viz., blue, others metachromatic, in the color of the base, viz., red. Metachromatic staining will produce the greatest contrasts on the fresh, unprepared tissue and is greatly influenced by our various fixing methods. A dye, in order to develop metachromatic properties, must generally be in watery solution, since alcoholic solutions show little or no metachromasia, the latter also being usually destroyed if the specimen is afterward treated with alcohol. A strong metachromasia and a decided basophilia may in many tissue elements go hand in hand, e.g., mucus, cartilaginous substance, certain cell granulations; on the contrary the nuclear chromatin is decidedly basophilic, but shows less metachromasia as does the greatly acidophilic collagenous connective tissue. The cell protoplasm is always orthochromatic. Definite knowledge of the cause of metachromasia is up to the present not claimed; perhaps the phenomenon is due to a tautomeric combination of the color base in question with the acid.

After this preliminary discussion of dyes and staining we will proceed to study singly the most important stains.

STAINS OF ANIMAL ORIGIN

Cochenille (Cocheneal)
Carmine.

The mother substance of carmine is the cochenille. The latter is a name given to the dried female of the Coccus Cacti, which habitates several species of the Opuntia Cacti in Mexico and Central America. The female insects are gathered from the plants shortly before shedding the eggs, they are then killed

in hot water and dried in the oven. Approximately 140,000 animals will yield 1 kg.[1] of cochenille.

Carmine.

Carmine is manufactured from the cochenille by extraction with alum and tartar and occurs as a deeply red, earthy, friable mass, being put into commerce in smaller or larger lumps. It is totally insoluble in water and in alcohol, soluble in alkalies, ammonia, acids, borax, and alum solutions.

Carminic Acid.

Chemically we must consider carmine as a combination of clay-lime-albumin with carminic acid. The latter is the coloring principle of carmine and in its pure state forms a beautiful red crystalline powder, readily soluble in water and alcohol. With alkalies it forms soluble, with earthy alkalies and the heavy metals insoluble salts.

In microtechnique the staining solutions are prepared from either cochinille or carmine, or carminic acid, as well. The best and most constant results are without doubt obtained from solutions made of chemically pure carminic acid, and for this reason we shall use such solutions almost exclusively.

Carmalum.

Make a hot solution of 5% potash alum, cool and filter; heat the filtrate and dissolve in it carminic acid to the extent of 0.5%. A deep bluish red solution results, which after cooling is filtered and preserved in 0.5—1% formalin. this solution will stain sections very rapidly and intensively, imparting a bluish red tone. After staining for usually ten to fifteen minutes the sections are placed in a 5% alum solution for a few seconds and then washed in water. In this way an almost pure staining of the nucleus is obtained. This stain is also adaptable to block staining. The blocks must not be too large and should remain in the staining fluid from twenty-four to forty-eight hours, after which they are washed in water.

Paracarmine.

This preparation is of still greater intensity and can be made by dissolving 1 gm of carminic acid, 0.5 gm of chloraluminum[2] and 4 gms of chlorcalcium [2] in 100 cm^3 of 70% alcohol, heating carefully. When the precipitate has settled, filter and keep in a well-corked bottle. For staining, this solution is diluted with five to ten times its volume of 70% alcohol, and acetic acid is added to the small extent of 1—2 drops to each. Paracarmine stains more rapidly and intensively than carmalum and can be used to the same advantage for sections or blocks. Alcohol, 70%, is used for washing; 2% acetic acid can be added to the alcohol, if an absolute pure staining of the nucleus is desired.

STAINS OF VEGETABLE ORIGIN

Flora furnishes us with dyes of great variety and number, which formerly were of decided importance to the practical dyer and are even to this day used

[1] Two pounds. [2] Chlorides.

frequently by him. For our purpose only two deserve mention: hæmatoxylin and indigo.

Hæmatoxylin.

It is an extract made from the wood of the hæmatoxylon campechianum, a cæsalpiniacea found in Central America, which is extracted in the form of small rhomboid, colorless crystals. It is not very soluble in cold water, but readily so in hot water, alcohol and ether. If a watery or alcoholic solution of hæmatoxylin is exposed to light and air, it will first assume a yellow tinge; later it will become brown.

Hæmatein.

The cause of this change in color is found in the fact that hæmatoxylin is wholly or partly oxidized to hæmatein. The latter is on the market in the form of a brown powder, which is soluble in water, alcohol, ether, glycerine, alkalies and ammonia in particular.

Hæmatoxylin *per se*, substantively, cannot be used as a stain; it is a typical representative of a mordant dye, forming, with most metal oxides, strongly staining lakes, their hue varying between blue and black. During the lake formation the hæmatoxylin is either at once or gradually oxidized to hæmatein, and the more advanced the oxidation is, the better the lake will stain. There is, of course, an optimum of staining power, which may and often is transgressed, since the oxidizing process does not stop with the formation of hæmatein, but will finally lead to the formation of oxalic acid. Hæmatoxylin lakes can be applied in either the simultaneous or the succedaneous method, i.e., we can stain with the ready solution of the lake or we may produce the lake in our specimen by successive applications of the mordant and the hæmatoxylin solution. To make the solution we may use either hæmatoxylin or hæmatein.

Hæmalum.

This is a clay hæmatein lake. Fifty grams of alum are dissolved in 1 litre of hot water, and the solution is cooled and filtered. One gram of hæmatein is dissolved in 50 cm^3 of 95% alcohol under gentle heating, and both these solutions are mixed. A dark bluish violet color reaction takes place at once. A sediment is common and renders it advisable to filter before using the stain. Hæmalum will stain sections in a very few seconds; the specimen is washed in water, which will impart to it a dark blue color. If overstained, a specimen can be reduced by being placed in slightly acidified water (2 drops of muriatic acid to 50 cm^3 of water). The latter solution extracts any superfluous dye, causing at the same time a change in color from the blue to a red tint. After being thus reduced the specimen is washed until it again assumes a blue color.

Iron Hæmatoxylin

probably furnishes a still stronger nuclear color result than the preceding stain; it is prepared as follows: 10 gms ammonium ferrosulphate, iron-alum, so-called, are dissolved in 150 cm^3 of hot water; in the same manner 1.6 gm hæmatoxylin are added to 75 cm^3 of hot water. Both solutions are cooled, one is poured into the other and the mixture is carefully heated over a small flame, stirring gently until the seething point is well reached. When cooled,

the solution, now dark brown, is ready for use. It stains very rapidly and thoroughly. If overstaining has taken place reduction is induced in the same manner as in hæmalum.

Hæmatoxylin-Iron-Alum.

Two solutions are used for this succedaneous mordant staining, which is generally spoken of as *Heidenhain* staining, after its discoverer: a 2.5% solution of ammonium ferro-sulphate (iron-alum) and a 1% solution of hæmatoxylin, which latter should be of some time standing and brown. The sections are first placed in the mordant for at least two hours or more, if necessary, after which they are washed for a short time and transferred into the color solution, where they remain overnight. At first they take on a gray color, later a grayish blue, then deep blue and finally they become entirely black. If reduction is necessary they should, after a brief wash, be replaced into the mordant, which gradually renders them lighter, with the appearance of black color clouds in the solution. This reduction or differentiation must be closely watched and should be controlled under the microscope. The nucleoli and the chromatin of the nuclei resist the extraction the longest, hence this procedure is a means of presenting these elements absolutely distinct. After the desired effect is obtained we transfer to a large vessel of hydrant water for at least ten minutes, changing the water repeatedly.

Hæmatoxylin-Potassium Dichromate,

a lake of hæmatoxylin, is used to differentiate the medullated fibres within the central nervous system, pieces of which are first saturated with the chrome salt, sections made thereof, and in these sections the lake is produced by introducing them into a hæmatoxylin solution. For further details of the method we refer the reader to the special part under Nervous System.

Hæmatoxylin-Phospho-Molybd c Acid.

Dissolve 1.75 gm of hæmatoxylin in 200 cm^3 of hot water, and add 10 cm^3 of a 10% solution of phospho-molybdic acid and 5 cm^3 of phenol (carbol. acid cryst.), previously liquefied by heating. Sections, previously treated with 10% phospho-molybdic acid for ten minutes and briefly washed in water, are kept in this dark blue solution for from fifteen to twenty minutes, washed in water and transferred to alcohol.

The entire specimen appears soft grayish blue, not very distinctly stained, but from this background the collagenous fibres contrast widely in their deep blue stain. By this method the connective tissue fibres are distinctly stained even in such places which are hard to bring out with ordinary stains.

Indigo.

A dyestuff known in the most ancient times, is found widely distributed in the floral kingdom, but its chief source is the Indigofera, a papilionacea cultivated in Bengal. We have here a very light, deep blue, friable mass, insoluble in water, alcohol and ether, but soluble in aniline, phenol and acetic acid. Concentrated sulphuric acid will dissolve it, with the formation of indigo-sulpho-acids. When treated with any reducing agent, indigo will be changed to indig-

white, which is soluble in water. The formation of indig-white and indig-sulpho-acid is responsible for the extensive use of indigo in commercial dyeing.

Indigcarmine.

In microtechnique we use a preparation produced by sulphuric acid acting as indigo, ***indig-carmine***, the sodium salt of indig-disulpho-acid, occurring as a deep blue powder, quite readily soluble in water, slightly or not soluble in alcohol. This preparation in a 0.25% solution makes an excellent plasma stain.

ARTIFICIAL STAINS

Artificial Stains.

Besides the coloring material furnished us by fauna and flora there is an abundance of others, artificially prepared, made synthetically by the chemist. They are therefore called artificial, although we must always bear in mind that all dyes, natural or artificial, can be traced back to a motherbody, benzol, so that we can take the latter point of view and deny any difference between them. Accordingly we have already succeeded in preparing synthetically some of the natural dyes, e.g., indigo.

Tar and Aniline Dyes.

All artificial dyes are made from substances contained in tar, and are therefore also known as ***tar dyes.*** The term "aniline dyes" is not comprising enough, since aniline is by no means the mother substance of all artificial dyes.

Varieties of Artificial Dyes.

A rational classification of artificial dyes according to their chemical composition gives rise to considerable difficulty. The substances in which we are interested are best divided into basic, acid and indifferent dyes.

Basic Dyes

Basic Dyes.

As basic dyes are known the salts of color bases, principally those of muriatic acid, but also the salts of sulphuric, nitric, acetic and oxalic acids. Most of them are more soluble in alcohol than in water. If an alkali is added to a watery solution of a basic dye, the color base will be thrown down, being, as a rule, insoluble in water. Tannic acid added to a basic dye will form insoluble tannate. If a basic dye in solution is treated with a strong reducing agent, decolorization takes place, a leuko-compound being formed, which may by oxidation be again transformed into the original dye.

Basic dyes possess an extraordinary affinity for the nuclear chromatin, for which reason they may be called ***nuclear dyes.*** Other structures affected by them preeminently are cartilaginous substances, granules of mast cells and mucus.

Fuchsin.

This is a nitric acid salt of rosaniline, and in commerce is also known as rubin, or magenta red, occurring in the form of small red crystals with a metallic lustre. It is prepared with an aniline water solution, an excess of the dye being suspended in aniline water (see p. 49) for several days under

repeated vigorous shaking. This solution will stain in from ten to fifteen minutes, acting more rapidly when warmed. After staining, the excess is extracted with 95% alcohol, to which 10% aniline may be added.

Methyl Violet.

The nitric acid salt of hexamethyl pararosaniline is a metallic shining crystalline powder, dissolving in water and alcohol with the production of a deep blue color. In microtechnique we generally use in its stead the impure ***gentian violet.*** This stain is also used in an aniline-water solution. It stains in from ten to fifteen minutes intensively. For washing we use first 95% alcohol, but when that seems insufficient, ***Gram's reduction method*** must be resorted to. One gram of iodine and 2 gms of potassium iodide are mixed with a few cubic centimetres of water and shaken until dissolved, after which enough water is added to make 300 cm^3. A few drops of this mixture are placed on the slide after decanting previous washing liquids, and are allowed to act until a brown color results, a few seconds being required; then the slide is quickly transferred to 95% alcohol. Here the slide will again assume a blue color, discoloration setting in at the same time. If this does not readily take place another treatment with the iodine solution should be given. By this method of decolorization, which is an important one in bacteriology, a wholly pure chromatin staining is made possible, giving excellent results, for instance in the recognition and study of nuclear changes.

Methyl Green,

a derivative of the previous dye, forms a green micro-crystalline powder, is easily soluble in water, not so readily in alcohol, giving a deep green color. One of our best nuclear stains, it is seldom used alone, but generally in connection with acid dyes.

Thionine.

Thionine, known also as *Lauth's* violet, is one of the oldest coal-tar dyes belonging to the thiazines. It occurs as a brilliant crystalline powder, having a metallic lustre; in water it will slowly dissolve with production of a blue-violet color. It is used in an 0.1% solution for nuclear staining. After staining (ten to fifteen minutes) we wash in 95% alcohol. Its principal importance lies in the fact that this dye stains metachromatically, e.g., principally mucus, cartilaginous substances and the mast cell granules; they are stained red, while the nuclei appear blue. If this metachromatic stain is to be maintained, no after-treatment with alcohol is permissible (see p. 78), but simple water is used for washing, and the specimen mounted in levulose.

Methylene Blue.

This most important of all coal-tar dyes is formed by the entrance of four methyl groups into the molecule of the preceding one. It occurs in various forms on the market. For our purpose the chemically pure crystallized methylene blue of the Hoechster Farbwerke is the most serviceable. It consists of small, glittering, brilliant crystallic needles, which are quite soluble in water and alcohol, to a small degree only is it soluble in normal saline, *Ringer's* solution, sea-water. If an alkali be added to a solution of methylene blue, the latter

is broken up into methylene azure and methylene violet. If a watery solution of methylene blue is treated with a reduction agent, it will be decolorized, the methylene blue changing to leuko-methylene blue. Reoxidation will take place, when the solution is shaken, by the mere action of the oxygen in the air. Living animal tissues, blood and urine will exert this same reducing action on methylene blue.

Methylene Blue for Nuclear Staining.

Methylene blue is an excellent nuclear stain. For such purpose it is used in a 0.5—1% solution; staining takes from ten to fifteen minutes, preferably in the warmth of the paraffin oven, after which the specimen is treated in 95% alcohol, to which 10% aniline can be added.

Methylene Blue in the Staining of Nerve Tissue.

The greatest value of methylene blue lies in its property of ***vital staining of nerves.*** Of the divers methods previously discussed, the following will give the best and most constant results for the central nervous system. As concerns the material, not all animals are equally well adapted to vital staining with methylene blue; rabbits, guinea-pigs and cats are most suitable. A 1% watery solution of the above-mentioned chemically pure methylene blue is filtered just before using, and heated to body temperature. The animal is anæsthetized with chloroform, the thorax quickly opened in the median line with bone shears, the pericardium slit and the left ventricle opened by cutting off the apex of the heart. The protruding blood is carefully aspirated in moist cotton and the thoracic cavity cleansed from blood coagula. We now search for the aorta, which emanates from the left ventricle closely behind the pulmonary artery and by means of curved forceps or, better still, an aneurism needle, we place a ligature loosely around this blood-vessel. As a cannula we use a glass tube, slightly bent in the middle, about 10 cm in length and 2—3 mm in bore. The end to be introduced is in the form of a bulb which terminates in a point, so that the ligature may not slip. Such a cannula can easily be made in any desired size (see p. 76). The point must be well polished or molten in a way so as not to injure the vessel wall. The other end of the cannula is attached to a rubber tube, which is closed by a glass cock or a pinch cock. The cannula as well as the rubber tube are first filled with *Ringer's* solution by aspiration, so that no air-bubbles may be contained within; the cock is then closed and the cannula introduced with the right hand into the root of the aorta by piercing through the left ventricle, while the left hand fixes the heart by means of mouse-toothed forceps. When the bulb of the cannula is seen lying in the aorta, an assistant will tighten the ligature placed previously, fixing the cannula in place. If no assistant is at hand, the cannula is introduced into the aorta as far as possible, then releasing his hold, the operator tightens the ligature himself. For an injection apparatus we may use a simple funnel suspended in a filter-stative. The funnel is connected with the cannula by a rubber tube. Fig. 25 shows an apparatus which is more efficacious. It consists of a graduated burette, provided with a stopcock, which is enclosed in a wide glass cylinder, the latter being closed at both ends with rubber stoppers.

A funnel is seen on top, through which the outer cylinder can be filled with warm water. Below an opening leads into a drain pipe, which is provided with a pinch-cock. The upper cork has a third opening for the exit of air, when the cylinder is being filled. The outer cylinder is filled with warm water, the burette with *Ringer's* solution, finally the lower end of the burette is opened to allow the fluid to escape and therewith expelling all air, after which it is attached to the rubber tube leading to the cannula, and all is ready for the injection. This has to be done slowly and without undue pressure. First the cannula-cock is opened, after which the one guarding the contents of the burette is released. All spurting vessels must be caught with forceps and tied off. Owing to the pressure caused by the return flow through the veins the right heart will become distended and must now be opened, which is best accomplished by cutting through the right auricular appendix. In this manner the entire vascular system is flushed with *Ringer's* fluid, until the latter, which is constantly taken up with cotton sponges from the right auricle, returns absolutely clear. In the case of a cat this will consume at least 300 cm^3 of the fluid. The burette is now filled with the staining solution and the injection proper begins. Here, too, we should work with the least pressure possible and slowly. The burette should be opened only so much as to admit a flow of 1—2 cm^3 per minute. During the entire duration of the injection the staining solution should be kept at a temperature of about 42°, which can be easily accomplished by draining off the water in the outer cylinder and replacing fresh warm water. As soon as the return flow from the right heart takes on a deep blue color, we tie or clamp the auricular appendix off, therewith closing the vascular system once more. It is hard to say how much of the stain should be introduced, at any rate we cannot very well overdo it—120—150—200 cm^3, when injected within two to three hours, should suffice for a large rabbit or a cat. After the injection is completed, the animal is left undisturbed for another half hour, and now the central organs are excised.

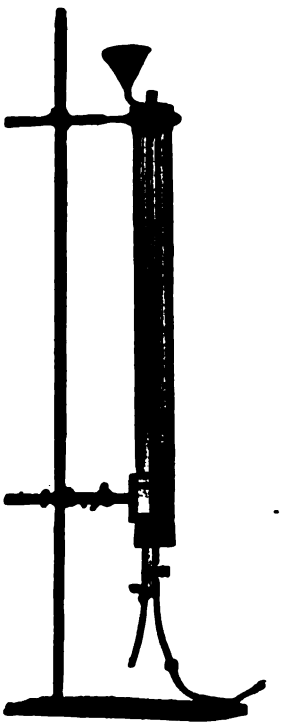


FIG. 25.

Apparatus for the Injection of Methylene Blue Solutions.

Protection of the Hands.

Since soiling of the hands with the dye is unavoidable during the injection, and more so during the ensuing preparation, it is a good plan to protect them by rubber gloves, such as are now in wide use in pathologic laboratories.

Reduction and Resulting Blue.

If the injection has been properly executed, the organs will appear perfectly colorless on opening the skull or the vertebral column respectively, but

as soon as the air comes in contact with them, they will assume a blue color, so that gray and white matter can be distinctly differentiated, the former appearing deep, the latter light blue. The organs should be sectioned into not excessively small pieces, say 3—5 mm in thickness and transferred immediately to the fixing bath.

Fixing of Methylene Blue Preparations.

We fix with a 10% solution of ammonium molybdate. The salt is pulverized in a mortar, dissolved in hot water, and the clear solution cooled well (8—5°). During the process of fixing, which should be carried on in the cool, the specimens will assume a still bluer hue. The molybdic acid forms a salt with the methylene blue base, which is readily soluble in water, but poorly in alcohol. Hence the specimens should on the following morning be thoroughly washed in running water for several hours, while the dehydration must be shortened and should be instituted under as low a temperature as possible. Four grades of alcohol suffice: 50%, 75%, 95% and 100%. In each of the first three mentioned the sections remain from three to four hours, being repeatedly shaken, after which they are placed in absolute alcohol overnight, which is changed once on the next morning. During the day they are transferred to xylol, in which they may remain for days.

To demonstrate the peripheral nerves it is necessary in most cases, and insures better success, to expose these tissues to the air for some time before fixing them, as such a precaution will bring out the optimum of nerve-staining. Coarse razor sections are made of the thicker structures, moistened with *Ringer's* solution and placed in a moist chamber. Now and then we make a control examination under the microscope. If after two hours no progress has been made in the nerve-staining, further waiting will be useless.

Organs which have been removed from the body or severed limbs can of course be injected in a similar manner.

Methylene-Azure.

This is the name of a dye which is produced by an alkali acting on methylene blue, being readily soluble in water with a blue color. It is seldom, perhaps never, used alone, but in conjunction with eosin (see p. 67).

Cresyl Violet R. B.,

an oxazin dye, coming into commerce as a bluish violet powder, dissolving with the same color production not so readily in water, but very easily in alcohol.

The importance of the dye lies in its metachromasia, which in this case is accentuated and finely shaded to such a degree that it is second to no other dye. A concentrated watery solution is made and for staining is diluted about ten times with distilled water. Staining will take place in from fifteen to thirty minutes, overstaining being impossible, even should thin sections be left for twenty-four hours. Metachromasia is evident most beautifully in the fresh specimen; of fixed material, frozen sections should be used exclusively. Formalin fixation is suited excellently, but sublimate and nitric acid give also good

results. After staining, the section is washed in water and mounted in levulose (see p. 79). A blue color will be imparted to the cell protoplasm, the interstitial granules of the muscle fibres, reddish violet to the nuclear chromatin, very light red to the collagenous tissue, deep red to mucus, cartilaginous substance, mast-cell granules, a yellowish red to the nerve-sheaths, a pure yellow to the hæmoglobin of the red blood cells.

Safranine.

Safranine O. Here we have a red powder, not very soluble in water, more easily in alcohol. A concentrated solution in aniline water (see p. 49) is prepared, in which the sections are stained for at least half an hour. To differentiate we transfer to 95% alcohol, to which a minute amount of hydrochloric acid may be added, a few drops of a 1% solution to 100 cm^3 of alcohol.

The metachromatic properties of safranine are not very pronounced. The best results are obtained on mucus, cartilage and sheaths of hair roots.

Resorcin Fuchsin.

This dyestuff deserves mention here, its chemical composition being little understood. Four grams of resorcin are added to 200 cm^3 of a 1% solution of fuchsin and the mixture warmed. When the boiling-point is reached, we add 25 cm^3 of liquor ferri sesquichlorati, which is immediately followed by a voluminous precipitate. The mixture is allowed to boil for a few minutes, is then cooled and filtered. The precipitate, a bluish black, muddy mass, together with the filter paper is brought to the vessel first used, 200 cm^3 of 95% alcohol are poured over it, the whole is heated to boiling over a water-bath, subsequently cooled and filtered; 4 cm^3 of hydrochloric acid are added and enough 95% alcohol to make 200 cm^3.

The bluish black solution so obtained will stain within fifteen to thirty minutes, elastic fibres will appear deep blue black, connective tissue a very weak blue, the basic substance of hyaline cartilage reddish violet. Excess of dye is washed out thoroughly with 95% alcohol.

Acid Dyes

Acid Dyes.

The dyestuffs considered under this heading are mainly the alkaline salts of color acids, with the possible exception of one staining acid, which must needs be mentioned here, namely, picric acid. All are invested with strongly pronounced acid properties, which are due to the presence of certain atom-complexes (NO_2, SO_3H, COOH, OH). Most of them are readily soluble in water and stain wool directly. Acid dyes may be decolorized by reduction agents, but a reoxidation thereafter is in most cases impossible.

While basic dyes show a pronounced predilection for the chromatin of nuclei, the acid dyes possess an affinity for protoplasm, for the achromatic portions of the nuclei and to connective tissue, to varying degrees; e.g., picric acid has a greater affinity for the cell protoplasm, while acid fuchsin selects the connective tissue for its action. For this reason acid stains are seldom used alone, but almost always in conjunction with basic dyes, either in mixtures or by using the succedaneous process. The mixtures will be spoken of later; as

regards succedaneous staining, the rule should be followed to stain first with the nuclear stain and secondarily with the acid dye, an excess of which latter can be extracted with alcohol, 70—95%. Often the acid dye will act as an agent of differentiation on the basic, so that the after-treatment with alcohol will extract more of the latter dye.

Picric Acid.

We have already been acquainted with trinitrophenol as a fixing agent; it plays a much more important part as a stain. Picric acid is an excellent plasma stain; its main use is for double staining after carmine and hæmatoxylin. We must, however, always bear in mind, that being a monobasic acid, it will strongly attract the nuclear stains of our specimen, similar to hydrochloric and acetic acid. It is necessary, therefore, to overstain in the nuclear dye, if a good double staining is desired. It is used in a 1% watery solution, staining cell bodies, connective tissue, muscle, red blood cells intensively yellow in a few minutes.

Acid Fuchsin

is the alkaline salt of rosaniline-trisulpho-acid, the latter being prepared by treating fuchsin with fuming sulphuric acid. It is a dark red powder, easily soluble in water, not quite so readily in alcohol. The watery solution is instantly decolorized by an alkali.

Acid fuchsin has attained great importance in histology, since it forms a component of many important staining mixtures. It is also very serviceable for staining after nuclear staining has been accomplished, a 0.05—0.1% solution in water being very efficient for this purpose. A possible overstaining can be corrected by washing in hydrant water. It plays an especially important part in the demonstration of *Altmann's* bioblasts.

Bleu de Lyon

(Lyon blue) is made also by sulphurizing a basic dye and occurs as a dark blue powder, readily soluble in water, more difficultly in alcohol.

It is well adapted for afterstaining of carmine preparations, for which purpose it is used in a 0.1% solution. Staining is continued until the sections begin to assume a blue color, when we transfer them into 70% alcohol. In well fixed specimens we can demonstrate nerves and differentiate them from the surrounding connective tissue by this method.

Light Green.

Light green is a dark green powder, readily soluble in both alcohol and water. It is used in the same manner as the preceding.

Orange G.

Orange G. is a representative of that large group of dyestuffs known as azo colors, which are of great importance in commercial dyeing. It forms small yellowish red crystal plates, which are soluble in water and to some extent in alcohol. Orange G. is one of our best plasma stains. It is especially used after hæmalum staining, in a 1% watery solution; it stains in a few minutes. The washing is done in 70% alcohol.

Bordeaux R.,

also an azo dye, is a reddish brown powder, readily soluble in water.

It is made use of in a 0.2% watery solution, which must be applied before the nuclear staining takes place; it stains in about five minutes, after which specimens are washed briefly in water and, as previously stated (see p. 57), are subjected to hæmatoxylin-iron alum. The more the differentiation advances the more will the red color due to the Bordeaux reappear.

Eosin.

Eosin is the sodium salt of a color acid, tetrabromfluorescin. In commerce it is found as a red powder, easily soluble in water. When a mineral acid is added to the watery solution, the insoluble color acid will be precipitated.

Eosin is extensively used as a plasma stain, especially following hæmatoxylin. The simplest manner of using it is to make a concentrated solution of the preparation called "eosin yellowish," and diluting the same with ten to twenty times its volume of water. The thinner the solution, the longer the staining will last. After staining we wash in 70% alcohol. Eosin is of great value in the staining of blood and blood parasites.

Indifferent Stains

Indifferent Stains.

Such is the name applied to a small group of dyes, the most important properties of which are their relative solubilities. They are totally insoluble in water, dissolve with difficulty in alcohol, but are very readily soluble in fats. Chemically these dyes are indifferent, i.e., they possess no salt-forming groups. Under this group belong, for instance, sudan III and scarlet R. (*Scharlach R.*), both of which are azo dyes. They are reddish brown or red powders, difficultly soluble in alcohol.

Sudan III and Scarlet R.
for Fat Staining.

A concentrated solution is made in 70% alcohol, the frozen sections are taken from water and placed in 50% alcohol for a few minutes, then into the staining fluid for fifteen to thirty minutes, after which they are washed in water. Fat will appear intensively red, likewise the sheaths of peripheral and central nerves. This process may be combined with a nuclear stain, e.g., hæmalum, as well as with a plasma stain, e.g., picric acid. In such a case we first stain with hæmalum, wash in water, transfer into 50% alcohol and thence into sudan; the specimen is then washed again in water, stained in a watery picric acid solution and finally rinsed with water. In the after-treatment we must of course avoid strong alcohol and all solvents of fat.

STAINING MIXTURES

Staining Mixtures, Homogeneous
and Heterogeneous.

As we have previously observed, multiple staining (excepting metachromatic staining) can be accomplished by either using the various dyes in succes-

sion or by staining with a mixture of the same. Three possibilities must be considered in the making of such a mixture: The component dyes may be basic, they may be acid, or they may partly be basic and partly acid. The first two varieties are easily prepared; such ***homogeneous*** mixtures are compounded of numerous basic and likewise a number of acid dyes. In the third variety, the ***heterogeneous*** mixtures, we are confronted with certain difficulties. If we mix the watery solutions of a basic and an acid dye, a reaction will take place, the color acid of the acid and the color base of the basic dye uniting to form a new dye, which we name neutral. These neutral dyes are generally insoluble in water, therefore a precipitate will be thrown down. We thus must expect a precipitate when mixing solutions of both acid and basic dyes. In order to bring such precipitate into solution we must employ other solvents, such as ethyl and methyl alcohol, acetone or methylal. Another way of procedure is to use an excess of the acid dye or to add an extra acid dye. What the nature of the solution is in the latter case cannot be stated with certainty, the probability, however, is that aside from the neutral dye it will also contain both the acid and the basic dyes in their original form.

Of the homogeneous mixtures only those of acid dyes are of interest to us; they are extensively used for afterstaining following nuclear dyes, in which case they will furnish an excellent differentiation between connective tissue and muscle. Sections stained with a heterogeneous mixture will show the chromatin of the nuclei, the basic substance of cartilage, mucus and the granules of mast cells in the basic colors, while all other elements will take up the acid dye or dyes, as the case may be.

Picrofuchsin.

Here we deal with a mixture of picric acid and acid fuchsin; it is prepared by mixing 45 cm^3 of concentrated watery picric acid solution with 5 cm^3 of a 2% solution of acid fuchsin. It is used after nuclear staining with hæmalum or iron-hæmatoxylin; it acts in five to ten minutes, the excess dye being extracted with 70% alcohol. Connective tissue will take on a bright red stain, while cell protoplasm and muscle will appear yellow.

Picro-Indigcarmine,

a solution of indigcarmine in picric acid, is prepared by dissolving 1 gm of the former in 300 cm^3 of a concentrated watery solution of the latter. This mixture is suitable for the after-staining of carmalum, paracarmine and safranine specimens. In five to ten minutes it will impart a brilliant blue color to connective tissue, cell protoplasm, and muscle taking on a grass-green hue. After staining, the specimens are washed in 70% alcohol.

Picrocarmine.

Its preparation necessitates magnesia water, which we can make by adding well water to magnesia usta, leaving this mixture stand for eight days, vigorously shaking it from time to time, so that we can always be sure to have an excess of magnesia present. Pulverized carmine (0.2 gm) is boiled in 100 cm^3 of magnesia water for half an hour in a flask loosely plugged with cotton. After cooling we filter and add a few drops of formalin. 100 cm^3 of a 0.5% watery solution of picric acid with 0.15 gm of magnesia carbonate are also

heated to boiling, cooled and filtered. The two solutions combined will give us the stain ready for use. Five to ten minutes will suffice to have nuclei stained red, all other structures taking a yellow stain.

Azureosin Solution.

This stain, also known as "Giemsa solution," contains 3 gms of eosin and 0.8 gm of methylene-azure dissolved in 250 gms of glycerine and 250 gms of methyl alcohol. One drop of the solution in 1 cm^3 of water will give a ready stain; its main use is for blood slides.

Biondi Solution.

This is the name given to a mixture containing one basic color, methyl green, and two acid dyes, acid fuchsin and Orange G. Its efficiency depends on the care taken in its preparation, which is as follows: The colors to be used should be ordered from the "Aktiengesellschaft für Anilinfabrikation" at Berlin, the following names being asked for: Methyl green N.M.P., acid fuchsin S.M.P., and Orange G.M.P. Of the former we take 3.4 gms, of the second 4.2 gms and of the third 3.0 gms; all three are placed in a small porcelain mortar and carefully triturated, so that a uniform powder results, which is dissolved in 100 cm^3 of distilled water. If a permanent solution is desired, the container must be made of the very best Jena glass. The best plan is to use an Erlenmayer flask, place in it the dry powder mixture, add the prescribed amount of water, cork well and, shaking it repeatedly, let the mixture stand on the paraffin oven for two to three days. After this period complete solution will have taken place, resulting in a dark brown fluid with a slight red tinge. This stock solution, when properly taken care of, will keep for a long time, and before using must be acidified and diluted. The following procedure, though approximate only, is to be recommended as practically very efficient for acidulation. Place 1 cm^3 of acetic acid in a 100 cm^3 graduated tube and add distilled water up to the 100 cm^3 mark, mix well and empty the glass; the traces of acid adhering to the wall will suffice for the acidulation. In a second smaller graduate 5 cm^3 of the stock solution are placed, and rinsing thoroughly with distilled water the contents are transferred to the acidulated tube, adding enough rinsing water to bring the solution up to 50, and now our stain is ready for use; it will keep well for a few days. As regards the dilution, one need not literally cling to the stated proportion. As far as the material is concerned, we must differentiate between frozen and paraffin sections. In the former case nearly all fixatives will yield good results, e.g., formalin, the various corrosive sublimate mixtures, nitric acid, also chrom-osmium-acetic acid, if the specimens have not been subjected to its action for too long a period. Of the paraffin sections only those fixed in sublimate or its mixtures are to be recommended as suitable. Celloidin preparations are only then admissable, if the celloidin has been extracted prior to the staining. The duration of the staining has little influence on the results; ten minutes will suffice; again we may stain for twenty-four hours with impunity. The staining is followed by a short wash with 0.5% acetic and the specimens are transferred into 70% alcohol, where thick color clouds will be seen to emerge. The use of the alcohol must not be protracted for very long, but as soon as the section takes on a red color, it should be put

through absolute alcohol and thence transferred to xylol. The results obtained are most excellent. A bluish green is imparted to nuclear chromatin, basic substance of cartilage and mucus; a bluish violet to the mast-cell granules; a red color appears in the nucleoli, the homogeneous juice of the nuclei, the centrioles, spheres and achromatic spindles, the cell protoplasm, collagenous and elastic tissues, contractile substances, oxyphilic cell granules, basic substance of bone, uncalcified dentine. The hæmoglobin of the erythrocytes will appear orange-colored. The shading of the red is so fine as to differentiate all various elements among themselves. In facility and simplicity of execution and variety of applicability this method is not excelled by any other. One disadvantage of *Biondi* solution stands out against its many valuable properties, the fact that specimens prepared in such manner will not keep indefinitely. This hampering objection, however, may be remedied by strictly observing the rules for finishing and mounting microscopic specimens, as laid down on pages 76–79.

APPENDIX TO METHODS OF STAINING

THE IMPREGNATION METHODS

Essentials of Impregnation.

Metal impregnation is the term applied to a process in which we aim to saturate a block or a section of our specimen with a solution of a metal salt, the latter undergoing such reduction in certain parts of the tissue, which enables it to form colored compounds or to be changed to the metal itself, respectively. Thus the metal might be precipitated in these places as fine granules, or these certain tissue elements may appear distinctly colored, without any precipitate being formed. In the latter case a differentiation between impregnation and staining is impossible.

Metals Concerned.

Of the metals used for this purpose only the noble metals and of these only silver and gold will interest us. Another impregnation, that with osmic acid, has been discussed among the fixation methods and impregnation in that case, when applied to fresh tissues, naturally serves as a fixation as well, since the salts of the noble metals will all coagulate albumin.

The metal salt solution may be allowed to act on either the fresh specimen or the fixed specimen or finally on sections made from the latter. Each of these three methods will answer the purpose.

The Reduction of the Metal Salt.

Reduction, the most important phase in the process of impregnation, may be induced without our aid by the tissue itself; ordinarily, however, we augment the process by the use of some aiding factors. One such factor is light—diffuse daylight or the direct rays of the sun. Under its influence the changes will take place much more rapidly and more intensely. Another such factor is found in acidulation; the reduction does not take place in distilled water, but

to the latter is added a greater or lesser amount of acid. Warming may in certain instances also hasten reduction. Finally we resort in many cases to measures such as used by the photographer in the developing of plates, namely, to the use of strong reducing agents, e.g., sulphurous acid, arsenious acid, formaldehyde, resorcin, aniline, pyrogallic acid, hydrochinon, etc.

Frequently we imitate the photographer so far as to follow our reduction with a hyposulphite of soda bath, to remove any metal salt which has not been reduced.

Impregnation with Silver Salts. Silver Nitrate.

Of the silver salts the only one of importance is silver nitrate. In commerce we find it either in the form of crystals or in sticks; it may be dissolved in water to the extent of 200%, forming a strongly corrosive fluid with neutral reaction. A stock solution of 20% strength should be made and kept in a clean, glass-stoppered bottle. If impurities are present, silver salt will soon be thrown down.

Silvering.

A solution of 0.5—1% (0.5—1 cm^3 of our stock solution to 20 cm^3 of water) will suffice to silver thin, membranous sections. The membranous tissue is taken from the animal, spread quickly on a flat plate, which is lined with paraffin and then fastened with hedgehog bristles; it is carefully rinsed just once with distilled water. According to the size of the specimen 10—20 cm^3 of the silver solution are poured over the sections and spread evenly by slight and even balancing over all the parts, which latter gradually become opaque. After ten minutes the solution is decanted, and we rinse in water several times, fill the plate with acidulated (1% acetic acid) water and expose it to light, preferably sun-rays, for reduction. The latter will take place very rapidly, the specimen becoming gradually brown. This silvering process preeminently brings out the cement-substance, being the classic method for that purpose.

Another use to which silver nitrate is put is the injection of the solution into hollow structures, such as the vascular system, lungs, etc. The technique will be described in the special part.

Chrome Silver Method.

The *Golgi* method, so called after its discoverer, consists in the successive treatment of the specimen with a chrome salt and silver nitrate solution. It is fair to assume that through the action of the bichromates on the tissues, chromalbumins are formed, the composition of which depends a great deal on the duration of the process. If the specimen is now brought into a silver nitrate solution, a compound of silver albumin-bichromate is formed in certain tissue parts, appearing black when viewed before a light. We will thus find these parts prominently black in a yellow field. Not all the tissue elements of the same kind are equally impregnated, since this process favors a certain amount of them, while it utterly disregards others. Besides this regular impregnation the entire surface of the specimen and some of the deeper parts will be found studded with granular or crystalline deposits, which act very disturbingly.

Rapid Chrome-Silver Method.

Of the many variations of the method the rapid method gives the best results, and we will therefore devote special attention to it. The fresh specimens are cut into small pieces and placed in a mixture of 8 parts of 2.5% bichromate of potassium and 2 parts of 1% osmic acid for two to five days; they are then dried with tissue paper and transferred to a 0.75% solution of silver nitrate, which must be changed after a few minutes. They will be ready for cutting the next day. The following details may well be observed:

Material for the Chrome-Silver Method.

Mostly every organ is suited for the chrome silver method, and in every one some part or other will be stained black: Connective tissue shreds, elastic fibres, muscle fibres, capillaries, neuroglia cells, nerve-endings, etc., but foremost among all others nerve-cells and their branches, peripheral and central nerve-fibres. The importance of this method rests with this property, and for this reason its main use is found in the study of the central and peripheral nervous system. The material should be as fresh as possible; however, we frequently find this requisite greatly exaggerated. Six to twelve hours after death we will still have tissues which give excellent results.

A more important fact is, especially when dealing with the central organs, that only very young specimens can be used. When human material is desired, infants one week old are to be recommended; of animals: mice, dogs, cats, two to ten days old, guinea-pigs at full term. The central organs of the calf are excellent, if they can be procured early enough.

Moderately sized pieces, not too small, are selected, perhaps 2—3 mm in thickness and 5—10 mm long. Several pieces should be taken from each specimen and all be placed in a brown vessel containing 50 cm^3 of fixing solution.

Duration of Impregnation.

It is of vital importance to know how long the pieces should remain in the solution. There are no set rules for each organ, the process being empirical. On the morning of the third day the pieces are dried superficially with filter-paper and thereafter placed in 10 cm^3 of silver solution, where a red precipitate of silver bichromate will instantly be formed. After a few minutes fresh solution is added liberally until the fluid remains clear. The following morning some razor sections are made and examined. If the impregnation is well established, the entire material can be silvered in like manner. If the result was not satisfactory, a few more pieces are selected and experimented on, etc. After seven or eight days a success is improbable.

Further Treatment of Impregnated Specimens.

Embedding can usually be omitted. The following is the best after-treatment. After remaining in the silver solution for from twenty-four to twenty-eight hours, the pieces are briefly rinsed in distilled water and in groups are placed at a proper height upon stabilit-blocks (p. 47), with a thick solution of gum arabic. After a few minutes the blocks are immersed in 70% alcohol,

after a few hours in a 95% solution of alcohol, where within a few hours they will attain the proper cutting consistency. The next day sectioning with the microtome may begin. The knife is placed at an obtuse angle and diligently moistened with 95% alcohol; the sections, which should not be too thin, are transferred to 95% alcohol. They are then transferred to carbolxylol (see p. 79), and finally to pure xylol, where they may remain a long time with impunity.

Mounting.

The mounting cannot take place in the usual manner, as will be described later; they will soon spoil if so treated. They are subjected to the following technique. Cover-glasses of corresponding numbers and sizes are cleansed and placed on a specimen board; the sections are taken from the xylol and placed on the cover-glasses, leaving a good margin of the glass free. As soon as the xylol begins to evaporate, a large drop of Canada balsam is placed on each section and the latter put in a dust-free place to dry. The following day the specimens are looked over and if any have risen in the balsam, another drop is applied. After a few days the specimens, now completely dry, are placed upon slides, which may be made of cardboard, wood, metal or glass, an opening being cut out in the place where the specimen is to rest (Fig. 26). Such slides can be bought, but on the other hand are very easily made from cigar-box wood with a little ingenuity. The cover-glass is placed in the opening, specimen side down, and secured with a little cement (see p. 77). If mounted in this fashion, specimens will keep for a long time and can even be examined with the oil-immersion lens.

FIG. 26.
Wooden Slide for Chrome-Silver Specimens.

The Pyrogallol Method.

We will give this name to the method, since, similar to the photographer, we use pyrogallic acid to create the silver image in the tissues. Specimens are placed in 90% alcohol overnight, 8 drops of spirits of ammonia (specific gravity, 0.91) having been added to each 100 cm^3 of alcohol; rinse with water and transfer to a 2% solution of silver nitrate for five to eight days, preferably in a thermostat to keep the temperature even. The pieces are now washed in water again and brought into the following reduction mixture: pyrogallic acid, 1 gm; formalin, 10 cm^3; water, 100 cm^3. After twenty-four hours they are dehydrated in the usual manner and embedded in paraffin. This method gives good results for the study of the neuro-fibrils in nerve-cells and fibres. It is best suited for the central nervous system, less efficient for the peripheral. The paraffin sections, mounted in the usual manner, can be gilded by placing them in a 0.1% gold chloride solution for several minutes, succeeded by ten minutes' treatment in 5% fixing soda, after which they are washed in water.

The Silver-Ammonia Method

can be used for frozen sections as well as for entire tissue blocks previously fixed in formalin. We will only discuss the latter. The blocks are washed in

distilled water for twenty-four hours, impregnated in a 2% silver-nitrate solution for six to eight days, quickly rinsed in water and placed in the following solution of silver-ammonia, which must be freshly prepared; 20 cm^3 of a 2% silver nitrate solution are placed in a large beaker with 8 drops of 40% sodium hydroxide, shaken and the resulting precipitate of silver oxide is dissolved by adding ammonia drop by drop until the solution is absolutely clear. The brown staining of tissues will be materially intensified by this solution. The following morning we wash once more and transfer to 20% formalin for reduction.

This method gives similar results as the one preceding. The collective staining of the connective tissue is a disadvantage.

Gold Chloride.
Impregnation with Salts of Gold.

Gold chloride is the salt used almost exclusively in microtechnique. In commerce we find it in the form of reddish-brown amorphous lumps or as yellow crystalline needles. The chemical composition of the former is $AuCl_3+2H_2O$, while the latter forms a compound with hydrochloric acid and is represented by $AuCl_3+HCl+3H_2O$. Both salts are highly hygroscopic, hence they are sold in sealed glass tubes. Of either one of the salts a 2% stock solution is prepared and kept in a well-stoppered white bottle. If cleanliness is observed, it will keep indefinitely. In the handling of all gold solutions metal instruments must be avoided, wooden sticks or glass needles being used in their stead; the latter can easily be made over the gas-flame from barometer tubes and drawn out to any desired shape or thickness.

Of all the numerous gilding methods, which have been advocated in the course of time, we will only discuss three.

Gold Chloride-
Lemon-Juice Method.

This method is especially adapted for thin organs, rich in connective tissue. The pieces, according to their thickness, are placed for from five to fifteen minutes in freshly expressed lemon-juice, which contains, besides its citrates, a goodly amount of free citric acid. The specimens will be found to swell considerably; they are now rapidly washed in water and placed in 1% gold chloride for a half to one hour. Again they are rinsed and then reduction is induced by placing them into 1% acetic acid, preferably on a white background and exposed to the direct rays of the sun. When taken from the gold solution the pieces present a light or dark yellow color; exposed to the light they will, after a shorter or longer period, assume first a dirty brown, later a red, and finally a bluish violet hue, depending on the intensity of the light. The last color mentioned might become so deep that the specimen appears black. Axiscylinders are most prone to be affected by the reduction, appearing, in a good specimen, distinctly black on a brilliant red background.

In order to make sections, the pieces are subjected to 5% formalin, and later are cut with the freezing microtome. Such preparations will last well.

Gold Chloride-
Formic Acid Method.

A process most suited to the demonstration of muscle nerves. Thin layers of the muscle are spread and fastened on wax plates and placed in 30% formic acid for ten to twenty minutes. We follow with 1% gold chloride for one-half to one hour, and finally reduce with the previously named acid in the dark. Reduction will be largely completed the next day. We now transfer to glycerine which must contain the same acid to the extent of 50% by volume, and substitute pure glycerine on the following day, which will act as a preservative.

Gold Chloride-Iodine-
Iodine-Potassium Method.

In conclusion we will deal with this process, which is used, not on fresh, but on preserved material, viz., sections. The gilding of such sections, whether frozen or paraffin, will always offer difficulties inasmuch as they must primarily be rendered susceptible to the gold salt. Frozen sections, fixed in any of the methods, are first placed in an iodine-iodine-potassium solution, made by diluting our laboratory solution (see p. 59) with an equal part of water, where they remain for thirty minutes. They will take on a prominent reddish brown. We now rinse them briefly in water and transfer them to a 0.2% solution of gold chloride for thirty minutes, where they are at first decolorized and later assume a yellow color. After washing again reduction takes place in a 2% solution of resorcine. Reduction will be complete in from one to two hours, but may be interrupted, if desired. Rinse in water and place in a 5% solution of sodium hyposulphite, which will, in the course of fifteen to thirty minutes, dissolve out all the unreduced gold. The sections are finally thoroughly washed. This method gives excellent results for many purposes, but chiefly serves to demonstrate connective tissue fibres, neuroglia giving the next best results.

METHODS OF INJECTION

As an appendix to general microtechnique the technique of injection deserves special reference at this juncture, a process which the microscopist has largely borrowed from the art of macroscopic procedure. By "injection" we understand the filling of any hollow structures of the animal body with foreign material by the aid of proper instruments.

The spaces may be of the most varied kind, e.g., the communicating channels of bony structure, joint cavities, and the excretory ducts of glands. As a rule, however, the term injection is applied exclusively to the filling of the blood and lymphatic systems.

Material Used for Injection.

As ***injection-material*** we can use all sorts of matter, which must needs be liquid (or gaseous) at the time of injection. For obvious reasons we select generally a colored injection material. They can be classified in different ways and may be spoken of as ***unstained*** and ***stained;*** the latter can be again divided into opaque and transparent. We can furthermore differentiate be-

tween ***liquid*** (i.e., remaining liquid) and ***coagulating.*** Liquid material is chosen for the injection of very fine blind ducts, such as the excretory ducts of glands, while coagulating fluids are better suited for blood-vessels.

Properties of an Efficient Injection Material.

When a colored mass is to be used, it must contain the particular color in an indiffusible form, so that it will not diffuse through the vessel-walls, staining the proximal tissues and thereby rendering the preparation useless. Hence dyes, which are insoluble in water, can always be used to advantage, provided we use a very fine suspension thereof. Soluble dyes give much nicer results, but of these only very few are not diffusible; here we can mention the soluble Berlin blue and the carmine, the latter, however, only under certain conditions.

Soluble Berlin Blue.

A concentrated watery solution of the soluble Berlin blue, previously filtered, makes the best watery injection material for glandular ducts.

For the injection of blood-vessels we always use a coagulating mass, i.e., a mass which is injected warm and liquid, coagulating when it becomes cooled. For this purpose a sufficient amount of glue is added to the color solution. Such a glue-mass coagulates easily and completely, and will not interfere with subsequent microtechnical procedures.

The Blue Glue-Mass.

An efficient blue glue-mass is prepared as follows: 50 gms of the best gelatine are soaked in distilled water for several hours; the water is thoroughly decanted at the expiration of this time and the gelatine placed in a beaker of 1,000 cm^3 capacity, and warmed over a water-bath until completely liquid. 500 cm^3 of a concentrated solution of Berlin blue of the same temperature are carefully poured into the gelatine, constantly stirring and raising the temperature until all of the dye has been dissolved. The hot mass is then filtered through flannel with the aid of a hot-water funnel.

The Red Glue-Mass.

A good red glue-mass is made by using 50 gms of gelatine, leaving it to swell in distilled water for several hours, after which time we decant the water carefully. The gelatine is now stained for two to three days in a solution of 15 gms of carmine in 2 *l* of 10% borax solution. The gelatine bars, after being soaked, should be transferred singly to the solution. After the staining the excess dye solution is washed off with hydrant water, and again the plates, now deep blue red, are singly transferred to a vessel containing 2% hydrochloric acid. The bars are agitated in this solution, until the bluish red changes to a cherry red. Subsequently they are freed from the acid by washing in running water; all water is removed or evaporated over the water bath. For purposes of preservation a piece of camphor, the size of a hazel nut, is added to the mass, while hot.

The Injection Syringe.

As an instrument for injection we recommend for most cases a strong metal syringe, holding 100—200 cm^3, the piston of which should have a washer of

leather or asbestos (Fig. 27). The anterior conical extremity serves for the attachment of an intermediate piece with stopcock, preferably a glass cannula, instead of the impractical metal cannula, as depicted in our illustration.

Fig. 27.
Injection Syringe.

Preparation of Glass Cannulæ.

These can be made in any desired form or size, to suit each particular case, in the following manner (Fig. 28). A glass tube of the proper calibre is selected and heated on one spot over a small gas-burner (microburner so-called), the tube being constantly rotated. When the glass has softened, it is drawn out according to how thin a cannula is desired. The flame is now turned very small and rotating, we again heat on the spot designated in Fig. 28 *a*, draw out slightly, thus molding the neck of the cannula; now we heat on the place shown in Fig. 28 *b* and draw out completely, so that the head terminates in a point. The point is now severed closely behind the head with the glass file and the opening polished smooth on a hone. The cannula shown in Fig. 28 *c* would answer better for many purposes if it would come to a finer point.

The cannula is connected with the intermediate piece by means of a strong rubber tube, which is secured on both ends with strong silk or thin wire.

To inject cold liquids the apparatus described on p. 61 can be utilized. By raising and lowering the entire apparatus, the pressure can be regulated at will.

Technique of Injecting.

It can be stated, as a rule governing all injections, that the smaller the area to be injected, the better will be the results obtained. Total injection of an entire animal body requires much care and experience. If a glue mass is to be injected, the specimen must have a temperature above the melting-point of the mass. Cold parts of a body must therefore be warmed for hours in water of 40—45°. When using recently killed animals, this is, of course, unnecessary. If the object is warm, we first introduce the cannula.

The Introduction of the Cannula.

A double, strong silken thread is placed around the vessel and left untied. After severing the latter, one-half is left in the hand of the operator to serve as a guide, while the assistant makes a loose loop of the other half. The operator now opens the vessel along its longitudinal axis to the extent of about 5 mm and introduces the cannula, which has previously been connected with rubber-tube and intermediate piece and filled with warm salt solution or, better

still, with an 8% solution of *Glauber* salt, all air-bubbles being expelled and the stopcock being closed at the time of introduction; as soon as the head of the cannula is within the vessel, the assistant secures it by tying the previously placed ligature. We can now inject our solution, which must be warmed to 40°, or we may first remove blood and clots by injecting a warm 8% solution of *Glauber* salt and then follow with the injection mass. By the latter procedure we warm our specimen and obtain a more complete injection; of course more of the mass is needed.

As soon as the injection material is seen to come from the efferent vessels,

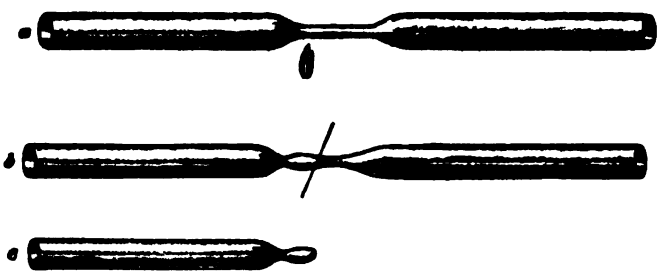


FIG. 28.
Preparation of a Glass Cannula.

the latter are closed with artery clamps (Fig. 29), and we continue carefully with our injection until the entire vascular system appears distended. It is

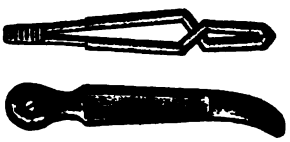


FIG. 29.
Artery Clamps.

a matter of practice and experience to know just when to discontinue the injection and how much pressure to use during it.

After-Treatment.

This is much the same as the routine with which we are familiar. The injection being complete, we cool the specimen in order to coagulate the solution, which may require hours in the case of voluminous organs. Smaller pieces are subsequently excised and fixed with any method desired, formalin *par excellence*.

MOUNTING AND FINISHING OF THE MICROSCOPICAL SPECIMEN

Durability of Stained Specimens.

After having stained our specimen according to one of the methods described, the question arises how to preserve it in a manner which is least apt to allow any changes to take place and will make the specimen a durable one. In

many cases this remains a hope, since among all our dyes there are few only of which it can be said that specimens stained therewith are absolutely lasting in color. Perhaps we can rightfully claim this for carmine and hæmatoxylin, but most all will be bleached in the course of years, and this is especially true of the artificial dyestuffs.

A great deal depends on the mounting and care of the slide thereafter. Any agent, to serve for mounting, must be chemically pure and above all must not contain a trace of any acid, which would destroy any stain in the course of time. Many artificial dyes being only slightly fast to light, specimens should be protected from light.

Mounting Media.

The agents used to enclose, i.e., mount our specimens, mounting media so-called, are partly liquid, partly solid. In the latter case they must naturally be liquefied by a proper solvent before using; by spontaneous or induced evaporation of this solvent the specimen, covered by the cover-glass, is thus enveloped in a layer of the solid medium, which serves also as a cement between slide and cover-glass.

Mounting in Liquid Media.

When using a liquid medium, the latter must be protected against evaporation, i.e., the specimen must be prevented from drying up, by the use of a strong cement between slide and cover-glass.

Cover-Glass Cement.

Such a cement can be prepared by melting 10 gms of wax in a porcelain dish and, while stirring, adding 35 gms of rosin. To make the mass more pliable a small amount of turpentine may be added. The mixture should be applied with a broad and not too thin spatula. The spatula should be warmed first and then dipped in the solid mass; the necessary amount of cement will thus adhere to the spatula and the latter can be heated to such a degree as to render the cement thinly liquid. The groove formed by cover-glass and slide is touched with the edge of the spatula, and thus a band of cement can be drawn, which solidifies at once. The surface to which the cement is applied must be absolutely clean and dry, lest the cement will not adhere. It is important therefore to use a small drop of mounting fluid, so that none will be protruding from under the edges of the cover-glass.

Mounting in Solid Media.

The process is simpler, when using solid media. A small drop of the liquefied medium should be used, for the smaller the drop, the thinner will be the layer enveloping the section. The cover-glass is grasped with forceps, the free edge is approximated until it touches the slide and the glass then slowly lowered, being careful not to enclose any air-bubbles. The specimen is then left to dry in a dustproof place. In certain instances it is of advantage to hasten the drying by the use of the thermostat.

Thickness, Size and
Cleansing of the Cover-Glass.

The cover-glasses with which we cover our specimens must be sufficiently large to cover the tissue entirely. The latter must never come close to

the edge of the glass, lest it shall be bleached more readily. This is especially true of hæmatoxylin preparations. The size of the cover-glass is naturally dependent upon the size of the slide, the latter generally measuring 26 × 76 mm, the so-called English type. In order to leave a narrow margin, the cover-glass must therefore not exceed 20 × 22 mm. Generally those measuring 20 mm square are well suited for histologic specimens, 20 × 40 mm for larger sections. The thickness should be of an average of 0.12—0.15 mm; thinner glasses are unnecessarily difficult to clean, while thicker glasses will not admit the use of the oil-immersion lens. They are best kept in a covered vessel, containing 30% alcohol. The cover-glass is taken out with forceps and dried with a clean cloth before using.

Influence of the Mounting Medium on the Microscopic Picture.

Of the innumerable media recommended for mounting we are interested in just three, glycerine, Canada balsam and levulose. Before going into details, we must make a few general remarks about their refractive properties. Our microscopic preparations possess a certain refraction index; if they should be placed in a medium with the same refraction index, all structural details will be lost, will be made invisible. Before being stained, the entire specimen appears equally illuminated. Staining will bring out the different hues in elegant fashion. If we desire to demonstrate the structural details in the unstained specimen, we must select a medium with low refractive index, hence unstained specimens are best to examine in a low refractive medium, e.g., water, which has a refractive index of 1.3. It is different with stained preparations, where the colors will not appear prominently enough in a low refractive medium; here it is better to select a medium with a higher index, say about 1.5.

Glycerine.

$C_3H_5(OH)_3$, a thick colorless fluid, mixing with water and alcohol in any proportion, is a good solvent for inorganic and organic substances. Its refractive index is 1.45. It is used a great deal as a mounting medium, but does not prevent the bleaching of the stain for any length of time. Pure glycerine, acting hygroscopic, will shrink the specimen; it is therefore advisable to use a mixture of glycerine and water first, and then follow with pure glycerine.

Canada Balsam.

This is made of the resin of several North American fir-trees, has a weak acid reaction and is soluble in ether, benzol, xylol and chloroform. The refractive index is 1.53. After the acid reaction has been neutralized by potassium carbonate, Canada balsam represents about the best mounting material for stained specimens known to this day. It should be dissolved in chemically pure xylol sufficiently to allow the solution to drop easily and be kept in glasses with a surmounting, closely fitting glass top.

Specimens to be mounted in balsam must first be thoroughly dehydrated by absolute alcohol and transferred to xylol. The slide is dried on its under surface and around the specimen, and a small drop of balsam is dropped on the section, which latter must never be allowed to become entirely dry. The cover-glass is now placed in the manner described above.

Celloidin sections must not be dehydrated in absolute alcohol, lest the celloidin should be softened too much. We therefore treat it with up to 95% alcohol, after which the small amount of water is extracted with carbol xylol, i.e., a solution of 1 part of phenol, liquefied by heating, in 3 parts of xylol. After the sections have become entirely transparent, they can be transferred to xylol and are then ready for balsam.

After a few days the xylol on the edge of the cover-glass will have evaporated sufficiently to make a strong connection between cover-glass and slide, while the balsam in the proximity of the specimen will remain liquid for months. It is to be recommended, especially in dealing with delicate stains such as *Biondi* solution, to place the slide in the thermostat for a few days immediately after it has been finished.

Levulose, Fruit Sugar.

The purest form is found on the market in form of light yellow crystals, readily soluble in water and alcohol to form a yellowish sirup; it has a refractive index of 1.5.

The only useful preparation is the crystallized levulose,[1] of which 20 gms are placed in a balsam glass containing 15 cm^3 of water, the glass to be kept in the paraffin oven overnight. Again we dry the cover-glass in the manner described above, trying, however, to withdraw any excess of water from the specimen itself by means of filter paper. A small drop of levulose is now placed on the specimen, which is left uncovered for one to two minutes. The cover-glass is now adjusted and the slide kept in the thermostat for several hours to allow the cover-glass to settle evenly; lastly we enclose with cover-glass cement. Levulose is an excellent mounting medium, possessing the advantage over balsam of dispensing with alcohol following the staining. It preserves better than glycerine, being inferior to balsam in this respect.

MENSURATION AND DRAWING OF MICROSCOPIC PREPARATIONS

In order to ascertain the actual size of the specimen seen in our microscopical image, we can proceed in different ways. We may either measure the specimen itself by the aid of proper devices or we can primarily project a true picture of it on paper by means of the drawing apparatus, measure this secondarily and, with due consideration of the magnifying power used, calculate the true size.

Ocular Micrometer.

Apparatus and devices for the measuring of microscopic specimens are called micrometers. They are constructed after various principles and in different forms. Simplest and most efficient for our purpose is the ocular micrometer. It consists of a small round glass plate, provided with scale; the scale is 5 mm long and divided into 50 equal parts. Special eyepieces are necessary for the use of the ocular micrometer, in which the eye-lens can be moved toward

[1] *C. A. F. Kahlbaum*, Berlin. Not inexpensive.

and away from the collective lens by means of a draw-tube. The latter is removed and, scale down, the micrometer is placed on the ocular diaphragm. When the eye-lens is replaced, we will thus obtain a magnified picture of the scale. The eyepiece being adjusted in the barrel, the images of scale and specimen will cover each other, since the former is projected through the eye-lens in the same plane as the latter. By moving the specimen we can place any desired field under the scale and measure how many of the subdivisions on the scale it will cover. Each segment of the scale has a certain value, which depends on the size of the image produced at the level of the ocular diaphragm, viz., it depends on the focal distance of the objective. The more powerful the latter, the smaller will be the value of each division of the scale. The optician furnishes tables with the micrometer, stating the values for the different objectives in a sufficiently accurate manner, so that we simply have to multiply the number of subdivisions with the value stated in the table. It goes without saying that during such calculations the prescribed barrel length must be strictly observed.

Drawing of Microscopic Specimens.

Of the significance and importance of drawing we have already spoken in the preface; suffice it to say that it is of the utmost importance that the beginner should draw his specimens repeatedly and in an exact manner. Most every beginner is possessed with a disliking for drawing, which we can often trace to an underestimation of the student's own faculties. The first attempt, if made at all, is generally such an utter failure, that the student becomes discouraged and fails to try again. For this reason it is well to aid the first attempts by the use of a drawing apparatus. Noticing how the picture develops under his hands by the simple tracing with pencil, he will acquire the ambition to complete the sketch, and after several such attempts he will have developed an interest and pleasure in this sort of work.

Drawing Apparatus.

Of the various makes of such apparatus we prefer the simple and cheap *Abbé* drawing apparatus (Fig. 30). It consists of two metal rings, the lower of which (r_1) is fastened centrically with three screws (*schr*) upon the upper extremity of the barrel; the other, upper ring (r_2), supports the apparatus proper and is connected with the first ring by a joint (*gel*), allowing of a forward movement of the apparatus. Upon the upper ring we find a small glass cube, enclosed in a little metal drum (*tr*), which is open on the side; the cube consists of two prisms, their diagonal planes being in approximation. The diagonal plane of the upper prism has been silvered mirrorlike, with the exception of a small central pupil of about 2 mm diameter. In front a horizontal arm (a) is seen emerging from the upper ring; at a distance of 7—8 cm a rectangular mirror is attached to this arm, which can be rotated on a horizontal axis. After the apparatus is properly attached, the mirror is adjusted at an angle of 45° to the arm, and now the observer, looking through the opening ($\ddot{o}$) in the drum and thence through the pupil of the prism, can see the microscopic picture and at the same time the point of a pencil, which is

held under the mirror, both pictures uniting in the eye, thus making it possible to trace the contours of the microscopic picture.

The use of the apparatus is not quite as simple as it may at first appear to be, since differences in light between the two images may result in one outbalancing the other. The apparatus being thrown back, we first focus our specimen sharply, and close the iris diaphragm; the point of the pencil must contrast on the white drawing paper. The diaphragm is now opened slowly, until specimen and pencil point appear equally distinct. Should the drawing surface be too light, which seldom is the case, smoked glasses should be interposed between prism and mirror. If we desire correct size, we must be sure to

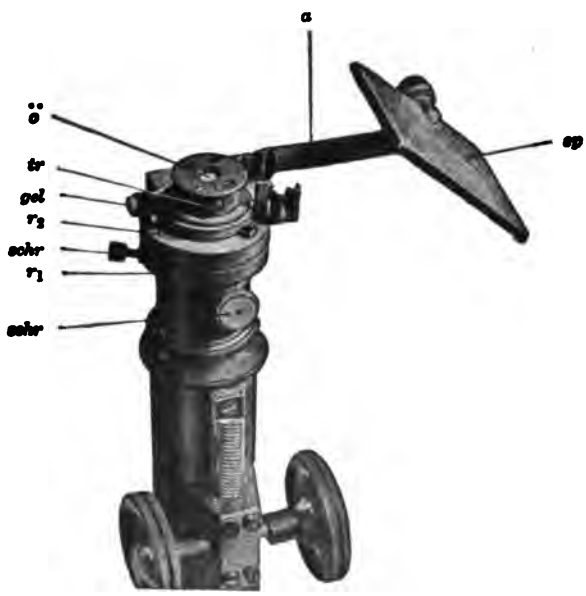


FIG. 30.

Drawing Apparatus after *Abbé*.

have the drawing surface on a level with the stage, and to avoid distortions our mirror must be kept at the angle of 45°. Only those places vertically under the mirror should be drawn; if larger drawings are desired, the drawing surface must be moved accordingly.

This apparatus serves only for the drawing of contours, the details must be worked in by free hand, reading from the microscopical picture. For this purpose we may use pencils of different strengths or, better still, a brush and india ink or colors. The drawing pen should be avoided, as it will impart a certain hardness to the picture which is foreign to the specimen. Of the brushes the Japanese are the best, results being obtained with them which are even more delicate than could be produced by the pen. Of the stains we would recommend the Gouache colors, manufactured by Schoenfeld & Co., in Düsseldorf. When minute details are desirable, it will be a good plan to draw through a simple lens, magnifying two to three times.

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